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Protein oxidation in meat: Effects on texture and water-holding

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ACADEMIC DISSERTATION

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‘博学之, 审问之, 慎思之, 明辨之, 笃行之’

——《礼记·中庸》

Learn extensively, inquire carefully, think deeply, differentiate clearly, and practice faithfully.

- Doctrine of the Mean / the Book of Rites

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Abstract

The aim of the thesis was to investigate the effects and mechanisms of protein oxidation on meat quality traits, such as texture, color and water-holding properties. To achieve this, meat was subjected to modified atmosphere packaging (MAP) with different oxygen concentrations and protein oxidation as well as meat quality traits (with a focus on texture and color) were measured. In addition, a model system of HClO-oxidized myofibrils was used to study the effect of protein oxidation on filament net charges, protein aggregation and their relation to water-holding.

Generally, protein oxidation was found to increase significantly both with increasing oxygen concentrations in MAP and with increasing concentrations of the oxidant HClO. Lipid oxidation, measured as TBARS, was also greater in meat stored under higher oxygen atmosphere.

Shear force of porcine *longissimus dorsi* (LD) muscle increased with increasing oxygen concentrations demonstrating a meat toughening effect. The role of proteolysis and protein cross-linking on oxidation-induced toughening was investigated. Myosin heavy chain (MHC) cross-linking was greater in 80% oxygen MAP than 0% and 20% oxygen, while desmin degradation was not affected by the oxygen concentrations in MAP, suggesting that oxidation-induced toughening was due to cross-linking of structural proteins rather than reduced proteolysis. Oxidation-induced toughening was also observed in minced beef as the hardness was generally greater in patties made from meat stored under 20-80% oxygen compared to 0% oxygen.

The internal redness of cooked patties decreased with increasing cooking temperatures and increasing oxygen concentrations in MAP, and a relative low oxygen concentration of 20% was able to cause premature browning (e.g., the patties made from 20% oxygen packaged meat showed brown cooked appearance at 55 ºC while 0% oxygen led to pink-red color).

In the model system, water-holding was generally improved by oxidation. Protein cross-linking was evidenced by increasing particle size with increasing concentrations of HClO. Isoelectric focusing (IEF) showed that the isoelectric point (pI) of solubilized myofibrillar proteins were generally lower following oxidation, indicating an increase of net negative charge. In the thesis, a hypothesis about oxidation-induced changes and their relation to water-holding is proposed: The oxidation-induced increase of net negative charges is due to loss of positively charged histidine residues through protein carbonylation, and water-holding is a balance between promoting factors (e.g., increased net charge) and inhibiting factors (e.g., cross-linking and aggregation).

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Helsinki, 07.02.2018

Yulong Bao

List of original publications

Paper I

Bao, Y., & Ertbjerg, P. (2015). Relationship between oxygen concentration, shear force and protein oxidation in modified atmosphere packaged pork. *Meat Science, 110*, 174-179.

Paper II

Bao, Y., Puolanne, E., & Ertbjerg, P. (2016). Effect of oxygen concentration in modified atmosphere packaging on color and texture of beef patties cooked to different temperatures. *Meat Science, 121*, 189-195.

Paper III

Bao, Y., Boeren, S., & Ertbjerg, P. (2018). Myofibrillar protein oxidation affects filament charges, aggregation and water-holding. *Meat Science*. 135, 102-108.

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I The study was planned by Yulong Bao and Per Ertbjerg together. The experimental analysis and manuscript preparation was done by Yulong Bao. Per Ertbjerg gave comments and suggestions during the manuscript preparation.

II The study was planned by Yulong Bao and Per Ertbjerg together. The experimental analysis and manuscript preparation was done by Yulong Bao. Per Ertbjerg and Eero Puolanne gave comments and suggestions during the manuscript preparation.

III The study was planned by Yulong Bao and Per Ertbjerg together. The experimental analysis (except that Sjef Boeren did protein identification with nanoLC-MS/MS) and manuscript preparation was done by Yulong Bao. Per Ertbjerg gave comments and suggestions during the manuscript preparation.

Abbreviations

AAS	α-amino adipic semialdehyde
ANOVA	analysis of variance
BPB	bromophenol blue
BSA	bovine serum albumin
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
CL-MHC	cross-linked myosin heavy chain
DNPH	2,4-Dinitrophenylhydrazine
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	1,4-Dithiothreitol
EDTA	ethylenediaminetetraacetic acid
GGS	γ-glutamic semialdehyde
HHE	4-hydroxy-2-hexenal
HNE	4-Hydroxy-2-nonenal
HSD	honest significant difference
IEF	isoelectric focusing
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LD	*longissimus dorsi*
MAP	modified atmosphere packaging
MDA	malondialdehyde
MES	2-(N-Morpholino) ethanesulfonic acid hydrate
MHC	myosin heavy chain
NS	not significant

pI	isoelectric point
PSE	pale, soft and exudative
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
TBARS	thiobarbituric acid reactive substances
TCA	trichloroacetic acid
TNB	2-nitro-5-thiolbenzoic acid
UPLC	ultra performance liquid chromatography
VP	vacuum packaging

1. Introduction

Meat proteins are susceptible to oxidation and oxidation-induced modifications on proteins may affect meat eating quality, protein functionality, nutritional value and link to aging-related diseases as summarized in Fig. 1. Protein oxidation in meat is currently an interesting topic in the food research area.

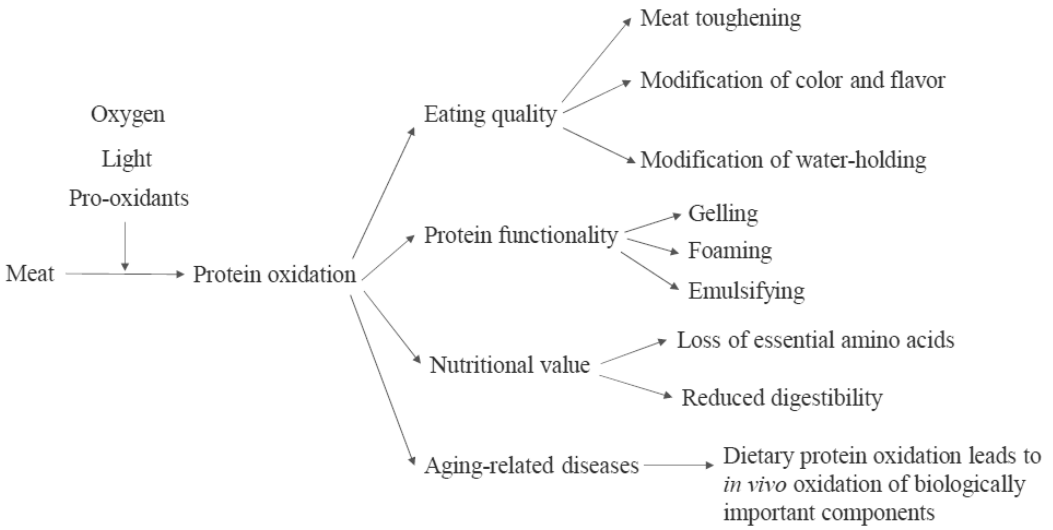

Fig. 1. Effect of protein oxidation on different aspects of meat quality.

Today, a major part of fresh meat is in many countries consumed after having been packaged stored under high oxygen atmospheres (70 - 80% O_2). In modified atmosphere packaging (MAP), a high concentration of oxygen functions to maintain the desirable bright red color in fresh red meat. The red color is desirable because consumers use meat color as an indicator of freshness. However, a high level of oxygen promotes oxidation of lipids and proteins. While many studies have revealed the effect of high oxygen packaging on meat quality (Clausen, Jakobsen, Ertbjerg, & Madsen, 2009; Jayasingh, Cornforth, Brennand, Carpenter, & Whittier, 2002; Lagerstedt, Lundström, & Lindahl, 2011; Lund, Lametsch, Hviid, Jensen, & Skibsted, 2007), much less is known on the relationship between the oxygen concentration in MAP, the oxidation process and meat quality.

Reduced sensory tenderness or increased instrumental shear force has been observed in meat subjected to oxidative conditions, such as irradiation (Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004a; Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004b) and high oxygen MAP (Lagerstedt, Lundström, & Lindahl, 2011; Lund et al., 2007; Moczkowska, Półtorak, Montowska, Pospiech, & Wierzbicka, 2017). Two mechanisms have been suggested for the increased toughness of meat after oxidation: Rowe et al. (2004b) suggested that oxidation can inhibit μ-calpain and result in less proteolysis of structural proteins which would explain the increased toughness; Lund et al. (2007) proposed protein-crosslinking as another mechanism for meat toughening. However, it remains unclear which of these mechanisms has the larger influence on meat texture.

Other than influence color of raw meat, high-oxygen packaging increases the incidence of premature browning in cooked meats (Seyfert, Hunt, Mancini, Kropf, & Stroda, 2004; Seyfert, Mancini, & Hunt, 2004; Suman et al., 2005). Premature browning is a condition that meat appear fully cooked (brown) while the internal part has not reached a safe temperature sufficient to kill possibly presented pathogens (Hague et al., 1994). Therefore, consumers need to be informed about premature browning in meat packaged in high oxygen concentrations as many would evaluate the doneness in meat by the internal color.

Some authors (Estévez, 2011; Lund, Heinonen, Baron, & Estévez, 2011) have argued that protein oxidation leads to decreased water-holding. However, no effect (Clausen et al., 2009; Lindahl, Lagerstedt, Ertbjerg, Sampels, & Lundström, 2010; Łopacka, Półtorak, & Wierzbicka, 2017) or increased water-holding (Chen, Zhou, & Zhang, 2015; Sekar, Dushyanthan, Radhakrishnan, & Babu, 2006; Yang et al., 2016) due to oxidation have also been observed. Therefore, the effect of protein oxidation on water-holding of meat, especially fresh meat, is unclear. Myofilament net charges are critical to water-holding (Hamm, 1972) and protein oxidation has been suggested to alter protein charges and subsequently influence water-holding (Estévez, Ventanas, Heinonen, & Puolanne, 2011; Utrera & Estévez, 2012), but more solid proof is needed.

In the present thesis, MAP with different oxygen concentrations (0, 20, 40, 60, 80%) and a model oxidation system (myofibrils oxidized with HClO) were used to study the relation between protein oxidation and meat quality traits, and the mechanisms of oxidation-induced changes in texture and water-holding were explored. The thesis first reviews the literature (Chapter 2) organized as: 1) a brief introduction of different reactive oxygen species (ROS) in meat and meat model systems; 2)

consequences of protein oxidation in meat at molecular level concerning side chain modifications, changes in protein charges, and cross-linking; 3) effects of protein oxidation on texture, water-holding and color; and 4) methods for investigation of protein oxidation in meat. The objectives of the present thesis are listed in Chapter 3. Materials and methods are briefly introduced in Chapter 4 and more details can be found from original publications. A summary of results of the thesis is given in Chapter 5 and they are discussed in Chapter 6. Finally, conclusions and future perspectives are provided in Chapter 7 and Chapter 8, respectively.

2. Literature review

2.1. Different reactive oxygen species in meat and meat model systems

Protein oxidation is believed to start from the abstraction of a hydrogen atom in the protein by reactive oxygen species (ROS). ROS is a collective term that includes oxygen-containing radicals ($O_2^{\bullet-}$, $^{\bullet}OH$, $RO^{\bullet}$, $RO_2^{\bullet}$…) and also some non-radical derivatives of oxygen (H_2O_2, HClO, O_3…). It is worth mentioning that reactive nitrogen species may also induce oxidative stress in muscle proteins (Skibsted, 2011).

ROS can be produced during normal metabolism of muscle and also upon exposure of meat to exogenous factors including oxygen in MAP, irradiation and chemical reagents. The nature of ROS is of importance for the resulting oxidative reactions. The hydroxyl radical ($^{\bullet}OH$) is very reactive and it can attack meat components in its vicinity, initiating oxidation in proteins and lipids. The hydroxyl radical can be produced by irradiation and also by the Fenton reaction, which involves a mixture of H_2O_2 and a transition metal. In meat and meat model systems, the H_2O_2-activated metmyoglobin can generate very potent oxidants (Baron & Andersen, 2002; Irwin, Østdal, & Davies, 1999). Oxidized lipids have also been used to initiate protein oxidation in meat (Park & Xiong, 2007; Xiong, Park, & Ooizumi, 2009) as radicals can transfer between different components (Schaich & Pryor, 1980). Although rarely used to study food, hypochlorous acid (HClO) is a strong oxidant that is able to mediate protein oxidation (Hawkins, Pattison, & Davies, 2003; Pattison & Davies, 2006; Pattison, Hawkins, & Davies, 2007). A schematic diagram summarizing HClO-mediated protein oxidation was given in Fig. 2.

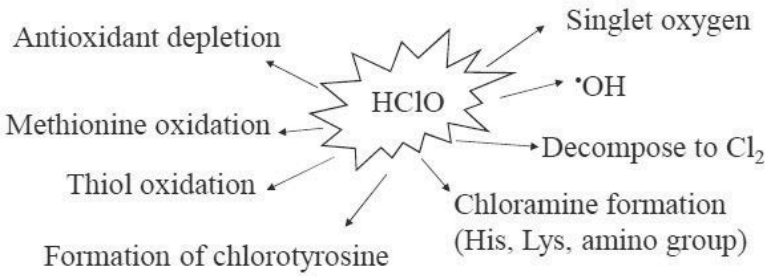

Fig. 2. A schematic diagram of HClO-mediated reactions which are able to modify protein molecules directly or by forming other reactive species. Modified from Pattison & Davies (2006).

HClO reacts with proteins leading to chloramines which can further produce nitrogen-centered radicals. Soglia, Petracci, & Ertbjerg (2016) found that HClO is very efficient in introducing protein oxidation in meat, as they observed marked increase of carbonyl groups in all fractions of meat proteins after incubation of minced meat with HClO. HClO is thus able to oxidize protein in meat systems to a much larger extent than a commonly used Fenton reaction, e.g. by overnight storage at cold temperatures (unpublished observation).

2.2. Consequences of protein oxidation in meat

In the research field of protein oxidation, many studies focused on the medical area owing to the relationship between oxidation-induced damages of proteins and age-related diseases (Berlett & Stadtman, 1997). Protein is one of the major components in food and therefore oxidation-induced damages to proteins, if accumulated to certain levels, is expected to affect food quality. Meat, being a protein-rich food and containing pro-oxidants, such as lipids and myoglobin, is susceptible to oxidation (Estévez, 2011; Lund et al., 2011). Oxidative conditions readily occur in post-mortem muscle during storage and processing and oxidative modifications of meat proteins at molecular level are introduced in this section.

2.2.1. Amino acid sidechain modifications

Essentially all amino acids can be oxidized (Davies & Dean, 1997). As reviewed by Lund et al. (2011), cysteine, tyrosine, phenylalanine, tryptophan, histidine, proline, arginine, lysine and methionine are particularly susceptible to ROS. Park & Xiong (2007) investigated the oxidative modifications of amino acids in myofibrillar proteins which were exposed to three different oxidizing systems (an iron-catalyzed oxidation system, a lipid oxidizing system, and a metmyoglobin oxidizing system). Cysteine was the only amino acid residue that decreased in all three oxidizing systems. The thiol group of cysteine residue is highly susceptible to oxidation and loss of thiol groups has been widely used as a marker for protein oxidation in meat (Delles & Xiong, 2014; Lund et al., 2007; Martinaud et al., 1997; Study I).

The formation of carbonyl is another one important side-chain modification in oxidized meat proteins. For a thorough review on protein carbonyls in meat, see Estévez (2011). The detection of specific carbonyls, lysine-derived α-amino adipic semialdehyde (AAS) and arginine- or proline-derived γ-

glutamic semialdehyde (GGS), enables in-depth study of the relationship between carbonylation and functionalities of myofibrillar proteins (Utrera & Estévez, 2012). Currently, the major protein-derived carbonyls in meat are regarded as AAS and GGS (Estévez, 2011). In addition to the amino acids resulting in AAS and GGS, other amino acids may oxidize into carbonyl products as well. The histidine-derived carbonyl, 2-oxo-histidine, has been detected in many proteins subjected to in vitro oxidation (Uchida, 2003); and also the amount of histidine in myofibrillar proteins was found to become lower when exposed to a metmyoglobin oxidizing system (Park & Xiong, 2007). Together, these studies suggest that it is worthy of investigation of 2-oxo-histidine in post-mortem muscle.

2.2.2. Oxidation and meat protein charges

The biological and biophysical properties of proteins are greatly affected by mobile electrolyte ions and by ionized fixed-charge groups attached to the backbones of protein molecules. It is critical to understand meat protein charges in relation to some of the meat quality traits. Oxidative modification of sidechains may lead to altered protein charges as described below.

2.2.2.1. Origin of protein charges

Several amino acid sidechains, the C-terminal carboxyl group and the N-terminal amino group of the protein backbone, can exist in basic or acidic forms, depending on their pK_a values and pH of the solution. The Henderson-Hasselbalch equation describes the relationship between pH, pK_a and the dissociation of a weak acid (Equation 1) or weak base (Equation 2).

$$pH = pK_a + \log_{10}\left(\frac{[A^-]}{[HA]}\right) \quad \text{Equation 1.}$$

$$pH = pK_a + \log_{10}\left(\frac{[B]}{[BH^+]}\right) \quad \text{Equation 2.}$$

The acid or base form that is prevalent at pH of 5.5 (around ultimate pH of meat) is marked in red in Fig. 3. Based on Equation 1 & 2, when the pH is less than the pK_a of a group, the protonated form of the group predominates. This leaves the acidic side chains with a charge approaching 0 and the basic side chains with a charge approaching +1. Conversely, when the pH is greater than the pK_a of a group, the deprotonated form predominates, giving acidic side chains a charge approaching -1 and basic side chains

a charge approaching 0. It should be noted that in folded proteins, the actual dominant form of amino acid residues also depends on the localized environment (Kuriyan et al., 2012). For example, a charged residue is energetically unfavorable in a hydrophobic environment but favorable when there is charged residues interacting with it.

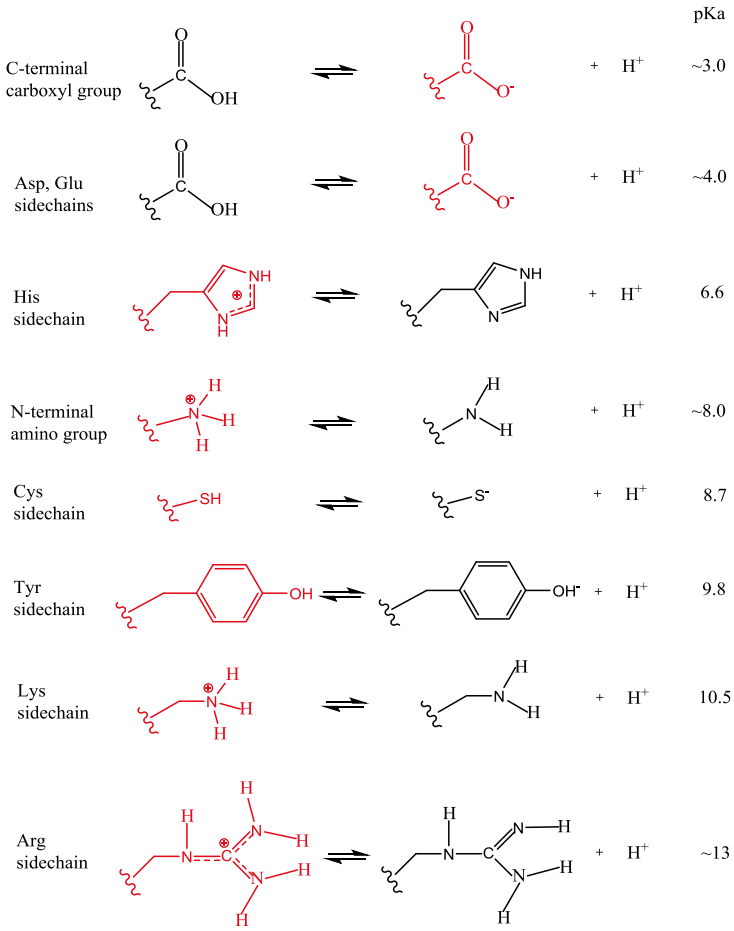

Fig. 3. The acid-base equilibria in protein groups. Shown here are sidechains and the terminal amino and carboxy groups of the backbone that can exist in charged forms, along with their pK_a values. The acid or base form that is prevalent at pH 5.5 is drawn in red color. The pK_a values given here are those for isolated amino acids, or unfolded sidechains. (Adapted from Kuriyan, Konforti, & Wemmer., 2012).

2.2.2.2. Charges at protein and filament level

Hamm (1972) studied the amount of positively and negatively charged groups of meat proteins as response to pH using a dye-binding assay and the results were presented in Fig. 4.

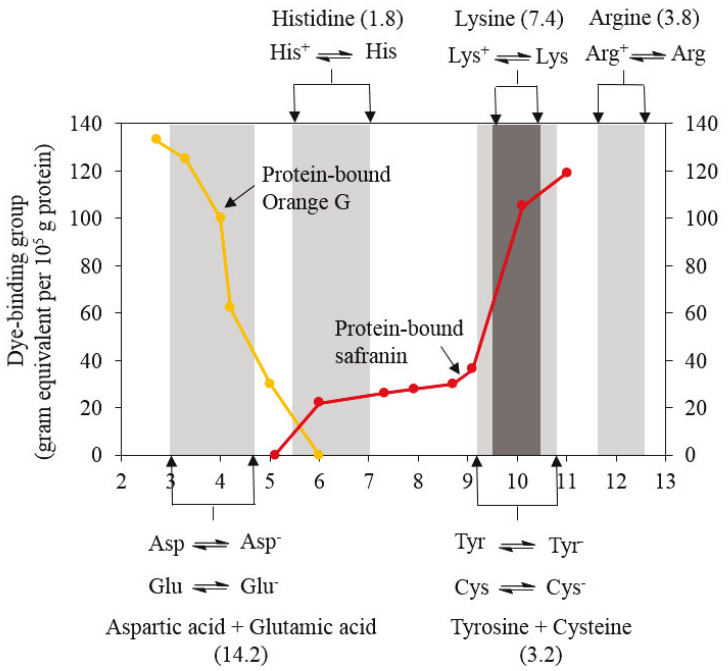

Fig. 4. Effect of pH on the number of dye-binding groups in myofibrils. Left curve: binding of orange G; right curve: binding of safranin. The equilibria of individual basic or acidic sidechains, together with their relevant pH ranges and amount in meat are given on the top or at the bottom of the figure. Changes in the amount and sign of net charges are indicated by protein-bound orange G (binds to positively charged protein) or safranin (binds to negatively charged proteins). The number in the brackets is the amount (moles / 10^4 g actomyosin) of the amino acids. Adapted from Hamm (1972).

Based on Fig. 4, at the pH of living muscle, aspartic acid and glutamic acid predominantly exist in the form with a charge of -1; histidine, tyrosine and cysteine remain neutral; lysine and arginine are in +1 charged form. This gives a net charge around -14.2 + 7.4 + 3.8 = -3 (mole / 10^4 g actomyosin). After slaughter when post-mortem muscle approaches the typical ultimate pH of 5.5, histidine will shift from being neutral to mainly positively charged, while other charged amino acid residues remain essential unaffected. This leads to a net charge of -14.2 + 7.4 + 3.8 + 1.8 = -1.2 (mole / 10^4 g actomyosin). As the

isoelectric point (pI) is where protein carries no net charges, it follows that the average pI of meat proteins is lower than 5.5. The value for net charges mentioned here is a rough estimation and other factors like bound ions have an effect as well.

While charges of protein groups depend on pH and pK_a, the net charge of a protein depends on pH and pI. The myofibrillar proteins as a whole has a pI around 5.0 based on the observations that water-holding is at its minimum (Hamm, 1972): Therefore myofilaments are negatively charged in fresh meat as the ultimate pH is generally around 5.5. The pI of muscle proteins can also be estimated by calculating the net charges on the muscle filaments from the average Donnan potential (Offer and Knight, 1988). According to Naylor, Bartels, Bridgman, & Elliott (1985), the Donnan potential together with the principle of electrical neutrality can give the fixed charges on the myofilaments. The measured Donnan potentials in rabbit psoas muscle in rigor changed sign at pH 4.5 in 10 mM KCl, or at pH 5.2 in 50 mM KCl. Therefore, the pI of meat proteins depends on ionic strength as well. In agreement, salt has been shown to affect pI of meat proteins (Hamm, 1972).

2.2.2.3. Effect of protein oxidation on protein charges

Protein carbonylation usually involves loss of lysine, arginine, and histidine residues (Stadtman & Levine, 2003) and these basic amino acids can exist in positively charged forms and, thereby, contribute to protein net charges. Therefore, protein carbonylation may modify protein net charges.

Davies, Delsignore, & Lin (1987) used isoelectric focusing (IEF) to detect alterations in the protein primary structure caused by oxidation. IEF gels separate proteins based on their pI and both positive and negative charge changes were observed with various proteins. Baraibar et al. (2011) reported that oxidative stress led to a shift of some proteins in human myoblasts to lower pI values.

In meat, protein carbonylation has been suggested to result in modified electronic arrangement on myofilaments (Estévez et al., 2011; Utrera & Estévez, 2012). Sun, Zhou, Sun, & Zhao (2013) studied the effect of oxidation on the emulsifying properties of myofibrillar proteins, and they found that oxidation led to changes in Zeta-potential of the emulsions which suggested a shift of pI to lower values. However, no direct measurement of protein charges following oxidation has been carried out in meat and more studies concerning the charges in meat at protein level or myofilament level are needed.

2.2.3. Formation of protein cross-links

2.2.3.1. Types of oxidation-induced cross-links

Formation of cross-links is one of the most common consequences of protein oxidation in meat (Lund et al., 2011). Protein cross-linking refers to the formation of covalent bonds within a protein (intramolecular) or between proteins (intermolecular). Several pathways lead to oxidation-induced protein cross-linking (Stadtman & Levine, 2003) and some of them are more relevant in meat systems as discussed in the following.

Formation of disulfide through oxidation of cysteine thiol groups

One of the oxidized products of cysteine is the disulfide cross-link (Fig. 5).

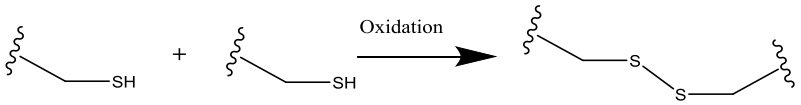

Fig. 5. Illustration of disulfide cross-link formation from cysteine residues.

SDS-PAGE results under reducing and non-reducing conditions have indicated the formation of disulfide-linked protein polymers in high oxygen packaged beef (Kim, Huff-Lonergan, Sebranek, & Lonergan, 2010; Study II) and pork (Delles & Xiong, 2014; Lund et al., 2007), as well as in chemically oxidized myofibril extracts (Decker, Xiong, Calvert, Crum, & Blanchard, 1993; Liu & Xiong, 2000; Xiong, Park, & Ooizumi, 2009). Disulfide formation is a common outcome of oxidative stress and many reactions lead to disulfides, such as interactions of thiols with sulfenic acids, conversion of thiosulfinate or thiolsulfonate esters, and radical-mediated thiol oxidation (Nagy & Winterbourn, 2010). The common strategy for detection of disulfides involves 3 steps: 1) blocking the free thiol group, 2) reducing the disulfides, and 3) detecting newly reduced thiols (Rysman et al., 2014). The disulfide bond is regarded as the main oxidation-induced cross-linking type in fresh meat and other types of cross-links present in meat as well (Lund et al., 2011).

Formation of dityrosine through interaction of two tyrosine radicals

Tyrosine is one of the primary targets for oxidation by various ROS, and it can be converted to dityrosine (Fig. 6), a specific marker for protein oxidation (Davies, Delsignore, & Lin, 1987; Giulivi & Davies, 1993). Formation of dityrosine has been reported in meat model systems (Bertram et al., 2007; Lund, Luxford, Skibsted, & Davies, 2008; Morzel, Gatellier, Sayd, Renerre, & Laville, 2006; Xiong et al., 2009) and very recently also in meat (Zhang, Li, Diao, Kong, & Xia, 2017). Bertram et al. (2007) showed that pH had a great effect on the formation of dityrosine and the formation was much higher at pH 5.5 than pH 6.2 and pH 7.0. Kato, Kitamoto, Kawai, & Osawa (2001) observed that dityrosine formed selectively in the H_2O_2/Cu system, but not in other metal-catalyzed oxidation systems. Similarly, Huang (2016) found that H_2O_2-induced oxidation of a model peptide (containing tyrosine) generated dityrosine, but not in ascorbic acid-induced oxidation. It is not clear if dityrosine can be a good marker of protein oxidation in meat due to limited available knowledge.

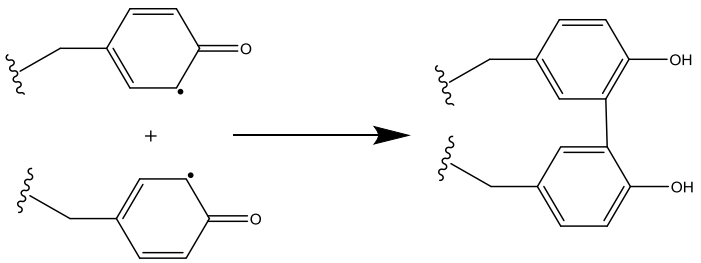

Fig. 6. Illustration of dityrosine cross-link formation from tyrosine-derived radicals.

Interactions of the carbonyl group with the primary amino groups of lysine residues

Protein carbonyls resulting from oxidation are reactive and Estévez (2011) reviewed the reactivity of protein carbonyls. Carbonyls may further react with lysine ε-amino groups which results in protein cross-linking (Fig. 7), or two specific carbonyls such as AAS may form an aldol condensation products leading to cross-linking (Fig. 8). The formation of carbonyl-amine condensation and aldol condensation products were supported by Estévez et al. (2011), where AAS and GGS of pork initially increased during frozen storage, but then followed by a loss of both semialdehydes, suggesting that carbonyls were invovled in protein cross-linking.

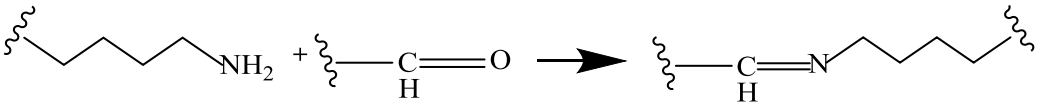

Fig. 7. Illustration of cross-link formation from reaction of a protein carbonyl with a lysine residue.

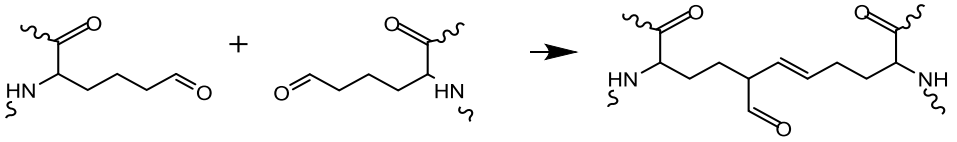

Fig. 8. Illustration of cross-link formation from reaction of two protein carbonyl (α-amino adipic semialdehyde, AAS).

Other than protein-derived carbonyls, lipid oxidation also generates carbonyl compound (such as MDA, malondialdehyde), which can react with ε-amino group of lysine residues in proteins. In theory, the oxidation reactions could be transferred between lipids and proteins (Zhang, Xiao, & Ahn, 2013). Reactions of both carbonyl groups of MDA with two different lysine residues would lead to protein cross-linking (Fig. 9).

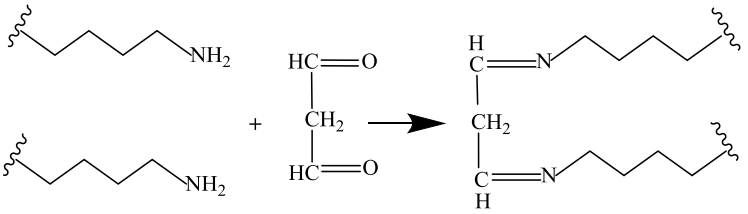

Fig. 9. Illustration of cross-link formation from reaction of MDA with lysine residues.

Buttkus (1967) showed that MDA reacted with myosin ε-amino groups and lysine was among the most reactive amino acid residues. Xiong et al. (2009) found an increased amount of protein-bound MDA in myofibrillar proteins exposed to different oxidizing systems, and they suggested that MDA contributed to the cross-linking of oxidized myofibrillar proteins. It has also been shown that other aldehydes than MDA are susceptible to form adducts with proteins, such as 4-Hydroxy-2-nonenal (HNE) (Aldini, Dalle-Donne, Vistoli, Facino, & Carini, 2005; Suman, Faustman, Stamer, & Liebler, 2006) and 4-hydroxy-2-hexenal (HHE) (Yamada et al., 2004).

In meat products, compounds from other sources are often added. Oxidative enzymes (e.g. tyrosinase, laccase) that have been used in meat model systems can facilitate formation of protein cross-links (Lantto et al., 2007; Lantto, Puolanne, Kruus, Buchert, & Autio, 2007). Some phenolic-rich plant extracts have been used as antioxidants in meat systems (Lund et al., 2011). Jongberg, Tørngren, Gunvig, Skibsted, & Lund (2013) found that addition of green tea extract to the recipe of Bologna type sausage enhanced protein polymerization and the polymerization was ascribed to interactions between quinone compounds from tea extract and thiol groups of meat proteins.

2.2.3.2. Cross-linking of structural proteins

In meat model systems subjected to oxidative stress, formation of protein aggregates were generally observed (Decker et al., 1993; Martinaud et al., 1997; Morzel et al., 2006; Santé-Lhoutellier, Aubry, & Gatellier, 2007; Xiong et al., 2009). Decker et al. (1993) incubated myofibrillar proteins in oxidative conditions and they observed marked loss of myosin and actin bands in SDS-PAGE gel (non-reducing conditions). The results indicated that myosin and actin formed large cross-linked products which cannot enter the gel. Martinaud et al. (1997) concluded myosin to be the most oxidizible among myofibrillar proteins. Xiong et al. (2009) investigated the cross-linking pattern of porcine myofibrillar proteins, and they found that the tail part of myosin was a favourable region for cross-linking.

Lund et al. (2007) studied effect of high oxygen MAP on protein oxidation of porcine LD muscle during chilled storage, and they observed cross-linked myosin heavy chain in high oxygen MAP but not in vacuum skin packaging, indicating that oxidation induced protein cross-linking. The loss of free thiols suggested that disulfide participated in cross-linking and this was confirmed by the observation that cross-linked protein bands disappeared under reducing conditions. By using 2D diagonal-PAGE, Kim et al. (2010) demonstrated an intermolecular cross-linking possibly between myosin heavy chain and titin.

Cross-linked myosin heavy chain has been identified as a major oxidation product in high oxygen packaged meat (Kim et al., 2010; Moczkowska et al., 2017; Rysman et al., 2014; Study I).

Collagen is a significant part of meat proteins, and knowledge about oxidation on collagen in post-mortem muscle is limited in the literature. It is generally accepted that collagen in the intramuscular connective tissue develops more mature cross-links as animal grow older (reviewed by Purslow, 2005), likely due to aging-related oxidation. Cross-linking of collagen has been shown when exposed to hydroxyl radicals (Kano, Sakano, & Fujimoto, 1987), ozone or UV radiation (Fujimori, 1988). In contrast,

fragmentation of collagen were reported in metal catalyzed oxidation (Uchida, Kato, & Kawakishi, 1992) and UV irradiation (Kato, Uchida, & Kawakishi, 1992). Protein backbone fragmentation is thus regarded as one of the common consequences of oxidation (Stadtman & Levine, 2003). Unlike cross-linking, which has been shown in meat and meat model systems, no clear evidence for oxidation-induced protein fragmentation has been found in meat. It should be noted that some would interpret the disappearance of proteins bands in SDS-PAGE gels as the result of fragmentation, however, this is more likely due to protein cross-linking.

2.3. Effects of protein oxidation on texture, water-holding and color

Color, texture and water-holding are important meat quality traits as they are linked to sensory acceptability and therefore the economic value. Several papers thoroughly reviewed the biochemical and/or biophysical basis for color (Mancini & Hunt, 2005; Suman & Joseph, 2013), texture (Bailey, 1972; Ertbjerg & Puolanne, 2017; Lonergan, Zhang, & Lonergan, 2010; Tornberg, 1996) and water-holding (Ertbjerg & Puolanne, 2017; Hamm, 1960; Huff-Lonergan & Lonergan, 2005; Offer et al., 1989; Offer & Trinick, 1983; Puolanne & Halonen, 2010) of meat. Those meat quality traits can also interact with each other and a structural approach to link those quality traits can be found in a review paper by Hughes, Oiseth, Purslow, & Warner (2014).

Protein oxidation readily occurs in post-mortem muscle. The impact of protein oxidation on texture, water-holding and color are introduced in this section.

2.3.1. Oxidation-induced meat toughening

Literature about the relationship between oxidative conditions and textural properties of meat with focus on MAP is summarized in Table 1 or meat model systems in Table 2.

Table 1. Summary of literature on the effects of high oxygen MAP on texture of meat.

Species and muscle[1]	Key observations	Reference
Lamb LD	Shear force →	Vergara & Gallego, 2001
Beef	Sensory score for texture likeness of beef patties ↓	Jayasingh et al., 2002
Beef LD	Tenderness ↓	Tørngren, 2003
Pork LD	Carbonyls→, Thiols ↓, Tenderness ↓, Sensory score for hardness ↑	Lund et al., 2007
Pork LD **Beef LD**	Myosin cross-linking →, Breaking strength of single muscle fibre of pork LD →, Breaking strength of single muscle fibre of beef LD ↑	Lund, Christensen, Fregil, Hviid, & Skibsted, 2008
Beef LD	Carbonyls →, Shear force →, Tenderness →	Zakrys, Hogan, O'Sullivan, Allen, & Kerry, 2008
Beef LD	Protein carbonylation ↑, Tenderness ↓	Clausen et al., 2009
Beef LD	Carbonyls →, Shear force →, Tenderness →	Zakrys, O'Sullivan, Allen, & Kerry, 2009
Lamb LD	Shear force ↑	Bórnez, Linares, & Vergara, 2010
Beef LL, SM, AD	Protein cross-linking ↑, In LL, Tenderness ↓ Compression force ↑ SM, AD →	Kim et al., 2010
Beef LD	Carbonyls ↑, Shear force →	Lindahl et al., 2010
Beef LD	Shear force ↑, Tenderness ↓	Lagerstedt, Ahnström, & Lundström, 2011
Beef LD	Carbonyls →, Shear force ↑, Tenderness ↓	Lagerstedt, Lundström, & Lindahl, 2011
Lamb LL	Protein cross-linking ↑, Shear force of older animal ↑ Shear force of younger animal →	Kim, Bødker, & Rosenvold, 2012
Beef LD	tenderness →	Resconi et al., 2012
Beef LD	Carbonyls →, Free thiols ↓, Shear force ↑	Zakrys-Waliwander, O'Sullivan, O'Neill, & Kerry, 2012
Lamb LD	Shear force →	Fernandes et al., 2014
Chicken PM, PL	Free thiols ↓, Protein cross-linking ↑, Tenderness ↓, Sensory score for firmness in PM ↑, Sensory score for firmness in PL →	Jongberg et al., 2014
Pork LD	Carbonyls ↑, Shear force ↑	Chen et al., 2015
Beef LD	Carbonyls →, Free thiols ↓, Shear force ↑	Fu et al., 2015
Lamb LD,SM	Tenderness ↓	Frank et al., 2017
Beef LL	Protein cross-linking ↑, Shear force ↑	Moczkowska et al., 2017
Beef TB	Compression force of myofibrillar gel ↑	Wang, Luo, & Ertbjerg, 2017

(Continued next page)

Table 1. (Continued)

Species and muscle[1]	Key observations	Reference
Pork LD	Free thiols ↓, Protein cross-linking ↑, Shear force ↑	Study I
Beef TB	Free thiols↓, Protein cross-linking ↑, Hardness for cooked patties ↑	Study II

1. AD *adductor*; BF *biceps femoris*; LD *longissimus dorsi*; LL *longissimus lumborum*; PM *pectoralis major*; PL *peroneus longus*; SM *semimembranosus*; TB *triceps brachii*.

'↑', increase; '→', no clear effect; '↓' decrease (High oxygen MAP compared to packaging with no oxygen or lower oxygen concentrations)

Although a few studies did not find a significant toughening effect due to oxidation, it is generally agreed that oxidative conditions, such as high oxygen MAP and irradiation, often lead to decreased tenderness or increased shear force of meat, as observed in beef, pork, lamb, and chicken. Some of the studies found that the toughening effect depends on animal species (Lund, Christensen, Fregil, Hviid, & Skibsted, 2008), animal age (Kim et al., 2012), and muscle type (Kim et al., 2010). The meat toughening effect is often accompanied by formation of protein cross-links (Kim et al., 2010; Kim et al., 2012; Moczkowska et al., 2017; Study I; Study II). Oxidation-induced textural changes were also demonstrated on single muscle fibre isolated from cooked meat samples (Lund, Christensen, Fregil, Hviid, & Skibsted, 2008) where breaking force of bovine LD muscle fibre increased in high oxygen MAP. This study shows the toughening effect was at fibre level. In myofibrillar gels, there is no general agreement on the relationship between protein oxidation and textural properties. The mechanisms involved in meat toughening are probably related to the oxidative modifications of proteins.

Table 2. Summary of literature on the effects of oxidative conditions on texture of meat and meat model systems.

Species and muscle[1]	Key observations	Reference
[a]**Turkey breast**	Carbonyls ↑ Myofibrillar gel strength ↓	Decker et al., 1993
[a]**Chicken breast**	Carbonyls ↑, Free thiols ↓, Protein cross-linking ↑ Storage modulus (G') of myofibrillar gel ↓	Liu, Xiong, & Butterfield, 2000
[a]**Pork SV**	Storage modulus (G') of myofibrillar gel ↑ Gel rupture force ↓	Xiong, Blanchard, Ooizumi, & Ma, 2010
[a]**Pork LD**	Carbonyls ↑, Free thiols ↓ Compression force of myofibrillar gel first ↑ and then ↓ with increasing oxidation	Zhou, Zhao, Zhao, Sun, & Cui, 2014
[b]**Chicken PS**	Shear force →	Heath, Owens, Tesch, & Hannah, 1990
[b]**Chicken breast**	Shear force ↑	Yoon, 2003
[b]**Beef LL**	Carbonyls ↑, Shear force ↑	Rowe et al., 2004a
[b]**Beef LL**	Shear force ↑	Rowe et al., 2004b
[b]**Minced camel meat**	Sensory score for texture →	Al-Bachir & Zeinou, 2009
[b]**Chicken PM, lamb BF, buffalo BF**	Shear force ↓	Kanatt, Chawla, & Sharma, 2015

1. BF *biceps femoris*; LD *longissimus dorsi*; LL *longissimus lumborum*; PM *pectoralis major*; PS *pectoralis superficialis*; SV *serratus ventralis*.

'↑', increase; '→', no clear effect; '↓' decrease ([a]chemical oxidation compared to non-oxidized control; [b]irradiation compared to non-irradiated control)

2.3.1.1. Role of protein cross-linking

The elastic pressure (P) of a gel is given by:

$$P = \frac{RT\ (v_e/V_0)}{q^{1/3}} \qquad \text{Equation 3}$$

Where q is the amount of swelling, R is the gas constant, T is the absolute temperature, v_e is the number of cross-linked units in the unswollen volume V_0. (Flory, 1953)

It can be seen from Equation 3 that the more cross-linked units, the higher the elastic pressure will be. Although muscle is much more ordered than a protein gel, there are sufficient general common principles involved. Muscle protein gelation contributes to the textural quality of comminuted fresh meat after being cooked. Wang et al. (2017) observed that oxygen in MAP led to cross-linked proteins in minced beef, and myofibrillar protein gels made from meat stored with oxygen were generally stronger and more elastic than without oxygen. Oxidation-induced cross-links may add to the heat-induced cross-links in the gel and therefore lead to increased gel strength.

Formation of cross-links in collagen is known to increase meat toughness (Purslow, 2005). Oxidation-induced cross-linking has been shown in collagen when subjected to UV radiation, ozone, and H_2O_2 (section 2.1.3.2). However, it is still unknown whether cross-linking of collagen occurs in postmortem muscle during MAP storage.

Both myofibrillar proteins and collagen contribute to the texture of meat (Bailey, 1972). Therefore it is reasonable to suggest that myofibrillar protein cross-linking contributes to texture changes of meat, similar to the effect of cross-linking in collagen. Lund et al. (2007) reported that high oxygen MAP led to formation of cross-linked myosin heavy chain (MHC) in pork together with a lower sensory tenderness. The observed larger particle size of oxidized myofibrils may also be induced by protein cross-linking. Myofibrillar protein cross-linking together with a decreased tenderness or an increased shear force due to high oxygen MAP is supported by other studies (Kim et al., 2010; Moczkowska et al., 2017; Study I).

2.3.1.2. Role of inactivation of enzymes

Calpains are believed to be involved in postmortem meat tenderization. Hydrolysis of peptide bonds by calpains requires a transfer of electrons between the active side chain cysteine and histidine residues (Mehdi, 1991). Both cysteine and histidine are susceptible to oxidation (Stadtman & Levine, 2003) and

therefore oxidation of the active cysteine or histidine residues could inhibit calpain activity and subsequently reduce the proteolysis of structural proteins and thereby lead to less tender meat. In addition to inactivation of the enzymes, oxidation by 1 – 5 mM H_2O_2 in the Fenton reaction may also negatively affect the susceptibility of myofibrillar proteins to proteolysis (Santé-Lhoutellier et al., 2007). In contrast, Smuder, Kavazis, Hudson, Nelson, & Powers (2010) claimed that oxidation (25 μM H_2O_2) enhanced myofibrillar protein degradation by calpain. These contrasting effects of oxidation on proteolysis may due to different degrees of oxidation: moderate oxidation may unfold protein structures while extensive oxidation often leads to more compact protein structures.

It has been shown that oxidation of purified μ-calpain resulted in loss of activity (Guttmann, Elce, Bell, Isbell, & Johnson, 1997; Lametsch, Lonergan, & Huff-Lonergan, 2008). In meat, evidence for oxidation-induced inactivation of μ-calpain has been reported by Rowe et al. (2004b) where the activity and autolysis of μ-calpain were decreased in irradiated beef samples. In agreement, it was found that high oxygen MAP inhibited the autolysis of μ-calpain and the proteolysis of desmin, a μ-calpain substrate (Chen et al., 2015; Fu et al., 2017). However, several other studies about high oxygen packaging found no effect on desmin degradation, indicating that the proteolysis of myofibrillar proteins was not affected (Kim et al., 2010; Lindahl et al., 2010; Study I).

2.3.2. Protein oxidation in relation to water-holding of meat

The relationship between oxidative conditions and water-holding properties of meat and meat model systems is summarized with focus on MAP is summarized in Table 3 or meat model systems in Table 4.

Oxidation-induced reactions are very complex and may interact with various water-holding determination methods where salt, pyrophosphate or cooking may be used in the procedures. In irradiated meat samples, water-holding generally decreased as compared to non-irradiated or lower-dose irradiated samples. In MAP with oxygen, currently there is no general agreement about the effect of protein oxidation on water-holding of fresh meat. Lund et al. (2007) found higher drip loss in high oxygen packaged pork as compared to vacuum packaged samples. Oxidation-induced decrease in water-holding is supported by other studies (Delles & Xiong, 2014; Zakrys-Waliwander et al., 2012). However, some stuides did not find a clear effect of high oxygen MAP on water-holding (Clausen et al., 2009; Lindahl et al., 2010; Łopacka et al., 2017) or an oppsite effect that vacuum packaged meat samples had higher drip loss or purge loss (Chen et al., 2015; Sekar et al., 2006; Yang et al., 2016). Traore et al. (2012a)

studied the relationship between drip loss and protein oxidation, and they found no difference in oxidised proteins of raw meat with different drip loss, suggesting that higher drip is not due to protein oxidation. Instead, pork samples with high drip loss are likely PSE meat, where protein denaturation is the main cause for poor water-holding. Under PSE conditions, endogenous antioxidant enzymes may be inactivated, therefore PSE meat is more prone to oxidation (Carvalho et al., 2017). This explains that during cooking (a process known to promote protein oxidation, Traore et al., 2012b), meat from high drip group showed a greater extend of protein oxidation (Traore et al., 2012a).

Table 3. Summary of literature on the effects of high oxygen MAP on water-holding of meat.

Species and muscle1	Key observations	Reference
Pork LD	Purge loss →	Sørheim, Kropf, Hunt, Karwoski, & Warren, 1996
Pork LD	Purge loss →	Cayuela, Gil, Bañón, & Garrido, 2004
Buffalo QF	Purge loss ↓	Sekar et al., 2006
Pork LD	Carbonyls →, Free thiols ↓, Purge loss ↑	Lund et al., 2007
Beef LD	Protein carbonylation ↑, Purge loss →	Clausen et al., 2009
Lamb LD	Expressible juice (filter paper compression) → Purge loss →, Cook loss →	Bórnez et al., 2010
Beef LD	Carbonyls ↑, Purge loss →, Cook loss →	Lindahl et al., 2010
Pork (ground)	Carbonyls ↑, Purge loss ↓, Hydration in salt and pyrophosphate ↑	Delles, Xiong, & True, 2011
Pork LD, PM	AAS ↑, GGS ↑ Water-holding ↓ (involves cooking and addition of salt)	Estévez et al., 2011
Beef LD	Purge loss ↓, Cook loss ↑	Lagerstedt, Ahnström & Lundström, 2011
Beef LD	Carbonyls →, Thawing loss ↑, Cook loss →	Lagerstedt, Lundström & Lindahl, 2011
Beef LD	Carbonyls →, Free thiols ↓, Purge loss ↑	Zakrys-Waliwander et al., 2012
Pork LL	Carbonyls ↑, Free thiols ↓, Centrifugation loss ↑ Hydration in salt and pyrophosphate ↑ Cook loss of oxidized and marinated meat →	Delles & Xiong, 2014
Beef LD	Purge loss ↑, WHC ↓ (filter-paper compression)	Bağdatli & Kayaardi, 2015
Pork LD	Carbonyls ↑, Purge loss ↓, Centrifugation loss ↑ T_2 relaxation time →, P_{21} (population of immobilized water) ↓ P_{22} (population of free water) ↑	Chen et al., 2015
Beef LL	Carbonyls ↑, Purge loss ↓	Yang et al., 2016
Beef LL,GM	Purge loss → (50%, 60% and 80% oxygen)	Łopacka et al., 2017
Beef LL, BF	Protein cross-linking ↑, Cook loss →	Moczkowska et al., 2017
Beef TB	Total water loss of myofibrillar gel ↑	Wang et al., 2017

1. BF *biceps femoris*; GM *gluteus medius*; LD *longissimus dorsi*; LL *longissimus lumborum*; PM *psoas major*; QF *quadriceps femoris*; TB *triceps brachii*.
2. '↑', increase; '→', no clear effect; '↓' decrease (High oxygen MAP compared to packaging with no oxygen or lower oxygen concentrations).

Table 4. Summary of literature on the effects of oxidative conditions on water-holding of meat and meat model systems.

Species and muscle[1]	Key observations	Reference
[a]**Turkey breast**	Carbonyls ↑, Centrifugation loss of myofibrillar gels ↑	Decker et al., 1993
[a]**Pork LD**	Dityrosine ↑,Water content of myofibrillar pellets → NMR T_2 relaxation time ↓ (implying reduced water-holding)	Bertram et al., 2007
[a]**Pork SV**	Centrifugation loss of myofibrillar gel ↑	Xiong et al., 2010
[a]**Pork LL**	Carbonyls ↑, Centrifugation loss ↑, Cook loss ↑ Hydration in salt and pyrophosphate ↑	Liu, Xiong, & Chen, 2010
[a]**Pork LD**	Carbonyls ↑, Tryptophan fluorescence↓, Cook loss ↑ Hydration in salt and pyrophosphate ↑	Liu, Xiong, & Chen, 2011
[a]**Pork LD**	AAS ↑, AAA↑, SB ↑ Centrifugation loss of heat-induced myofibrillar gels ↑	Utrera & Estévez, 2012
[a]**Pork LD**	Carbonyls ↑, Free thiols ↓ Centrifugation loss of myofibrillar gels ↓ and then ↑ with oxidation	Zhou et al., 2014
[a]**Pork LD**	Carbonyls ↑, Free thiols ↓, Centrifugation loss ↓	Study III
[b]**Chicken**	Expressible juice (filter paper compression) ↑ Centrifugation loss ↑ Water-holding of heat-induced myofibrillar gel ↓	Zabielski, Kijowski, Fiszer, & Niewiarowicz, 1984
[b]**Chicken PS**	Cook loss of aged samples → Cook loss of non-aged samples ↓	Heath et al., 1990
[b]**Pork LD**	Purge loss ↑	Lambert, Smith, & Dodds, 1992
[b]**Chicken breast**	Cook loss →	Yoon, 2003
[b]**Goat legs**	Expressible juice (filter paper compression) ↑	Modi et al., 2008
[b]**Chicken PM, lamb BF, buffalo BF**	Cook loss ↑ Centrifugation loss of minced meat/salt mixture ↑	Kanatt et al., 2015

1. BF *biceps femoris*; LD *longissimus dorsi*; LL *longissimus lumborum*; PM *pectoralis major*; PS *pectoralis superficialis*; SV *serratus ventralis*.

2. '↑', increase; '→', no clear effect; '↓' decrease ([a]chemical oxidation compared to non-oxidized control; [b]irradiation compared to non-irradiated control).

Unlike purge loss or drip loss, hydration of muscle pieces in the presence of salt and pyrophosphate are generally better with oxidation (Delles & Xiong, 2014; Liu et al., 2010; Liu et al., 2011). In model oxidation systems, centrifugation loss of myofibrillar gels generally increased with oxidation (Decker et al., 1993; Liu et al., 2010; Utrera & Estévez, 2012; Xiong et al., 2010), but Study III showed a decreased centrifugation loss of HClO-oxidized myofibrils. Most water in muscle is held within the myofibrillar protein matrix (Huff-Lonergan & Lonergan, 2005). The volume changes of myofibrils has been suggested as a good mechanism for changes in water-holding observed in meat (Offer & Trinick, 1983). Myofibrils are composed of sarcomeres and the unit volume of sarcomere is determined by sarcomere length and lattice space. Recently, a detailed review paper has discussed the structure of the sarcomere in post-mortem muscle and its relation to water-holding (Ertbjerg & Puolanne, 2017). In post-rigor raw meat, swelling or shrinking of myofibrils mainly happens laterally (changes in lattice space). Filament net charges and structural constraints are two important factors that influence the lattice space (Smith, 2014) and thereby water-holding of meat.

2.3.2.1. Role of filament net charges on water-holding

As explained in section 2.2.2.2, myofilaments are net negatively charged in postmortem muscle and the electrostatic forces tend to push the filaments apart. Hamm (1972) concluded that the electrostatic repulsion between myofilaments contributes to water-holding of myofibrils. In addition to electrostatic repulsion, filament net charges could result in an uneven distribution of counter-ions being more concentrated near the filaments and thus creating osmotic forces leading to swelling pressure of myofibrils (Offer & Knight, 1988). Both Hamm (1972) and Offer & Knight (1988) hypotheses support that increased filament net charges lead to increased water-holding, and explain the effects of salt content and pH on water-holding (Puolanne & Halonen, 2010). Typical effects of pH and salt on water-holding is given in Fig. 10 and the corresponding mechanism in Fig. 11. Myofibrillar proteins on average has a pI around 5.0 where filaments carry minimum net charges and therefore a minimum lattice space. Filaments gradually become more positively charged at pH below the pI, while they are negatively charged above pI. Increased net charges lead to larger filament space (Fig. 11) and better water-holding. The addition of salt shifted the pI from near 5.0 to 4.0, which may be due to a preferred association of Cl^- (than Na^+ ions) with myosin filaments as the chaotropic chloride ion would probably be absorbed to the hollow and hydrophobic part of myosin filaments in addition to the outer surface (Puolanne &

Halonen, 2010). The addition of salt, depending on pH, thus have negative or positive effect on filament net charges and thereby water-holding as shown in Fig. 11.

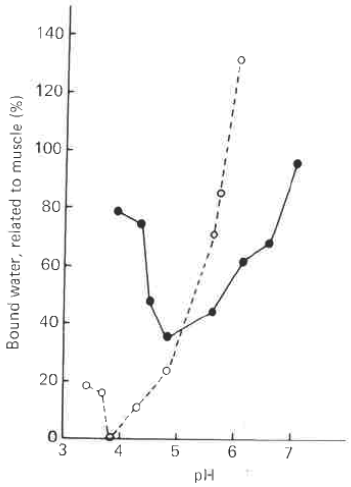

Fig. 10. Influence of pH and salt (2% NaCl) on the water-holding capacity of comminuted beef (50% added water, filter-paper press method). Empty circle represents the salted sample. Hamm, 1986.

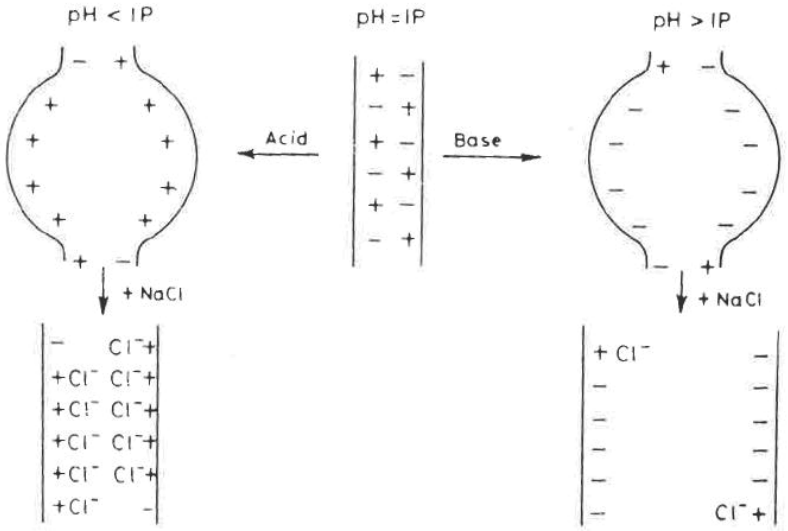

Fig. 11. Scheme of the influence of pH and salt on swelling of meat (pI, isoelectric point), two adjacent myosin filaments are shown Hamm, 1986.

Filament charges may also play a role in effects of sarcoplasmic protein denaturation on water-holding. Liu, Arner, Puolanne, & Ertbjerg (2016) observed a decreased distance between filaments upon denaturation of sarcoplasmic proteins. As the pI of sarcoplasmic proteins are generally between 6 and 7, they will have net positive charges at ultimate meat pH of 5.5. Those authors suggested that denatured sarcoplasmic proteins precipitated onto myofilaments and added positive charges to filaments and thus decreased the net negative charges, which could explain the observed decrease of filament spacing and water-holding.

According to Collins (1997), stable charges such as of biomolecules are strong chaotropes, such as basic amino acid residues: lysine, arginine and histidine. The structure-breaking chaotropes may make water molecules less structured and induce high density water. The high density water is more mobile and therefore is hypothesized to reduce water-holding (Puolanne & Halonen, 2010).

Lysine, argine and histidine are prone to oxidize into carbonyls. Carbonylation of those positively charged amino acids would lead to a loss of positive charges. In meat, lysine- and arginine-derived carbonyls have been used as a biomarker for protein oxidation (Estévez, 2011). The histidine content of myofibrillar proteins was found to decrease with increasing oxidation in a metmyoglobin oxidizing system (Park & Xiong, 2007). A histidine derived carbonyl, 2-oxo-histidine, has been detected in many *in vitro* oxidized proteins (Uchida, 2003) while more recently Study III suggested the formation of 2-oxo-histidine in oxidized myofibrillar proteins. It has been suggested that oxidative modification of the amino acid side chains lead to modified electronic arrangement on myofilaments and the modification of filament charges affect water-holding (Estévez et al., 2011; Utrera & Estévez, 2012). Sun et al. (2013) observed a shift in the isoelectric point of emulsion droplets towards a lower pH in oxidized myofibrillar protein samples. If pI shifts towards a lower pH, water-holding is expected to increase as in fresh meat pH is normally higher than pI.

2.3.2.2. Role of structural constraints on water-holding

Protein oxidation may inhibit proteolysis in meat (section 2.3.1.2) and Huff-Lonergan & Lonergan (2005) suggested that oxidation-induced inhibition of proteolysis negatively affects water-holding. A link between post-mortem proteolysis of cytoskeletal proteins and water-holding was proposed by Kristensen & Purslow (2001) as illustrated in Fig. 12.

From stage A to stage B, myofibrils shrink due to rigor onset and water flows out the muscle fibre; from stage B to stage C, inter-myofibrillar and constameric linkages are degraded and a loosened structure allows water to flow back from extracellular to intracellular. Therefore, oxidation may inhibit the proteolysis process from stage B to stage C and lead to impaired water-holding.

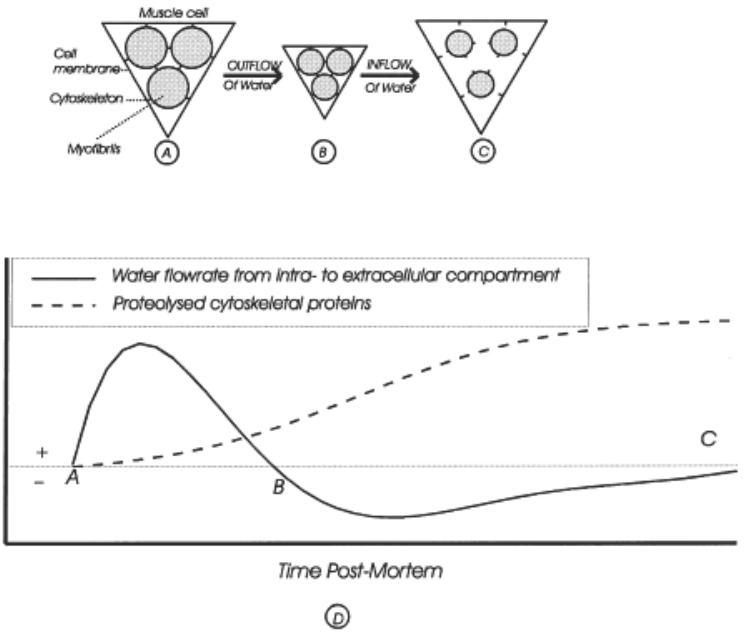

Fig. 12. Hypothesis for increased water-holding due to proteolysis. (A) A simplified pre-rigor muscle fibre with three myofibrils connected to each other and also the sarcolemma. (B) Shrinkage of myofibrils leads to a shrinkage of whole muscle fibre. (C) Proteolysis removes the linkages between myofibrils and sarcolemma. (D) Relation between water flow rate, time post-mortem and proteolysis. Kristensen & Purslow, 2001.

Farouk, Mustafa, Wu, & Krsinic (2012) proposed another mechanism for improved water-holding during aging. Proteolysis of meat structural proteins disrupts the drip channels thereby creating a sponge-like structure and some water is physically entrapped in meat and the amount of drip is reduced. Protein oxidation may reduce the sponge effect due to less proteolysis.

Swelling or shrinking of myofibrils has been suggested as the mechanism for changes in water-holding (Hamm, 1960; Offer & Trinick, 1983). In the abovementioned two hypotheses, myofibril swelling or shrinking is not involved in relation to proteolysis. In meat, there are transverse elements (Z-disks,

actomyosin cross-bridges and intermediate filaments) which resist the swelling or shrinking of myofibrils thereby affecting water-holding. Recently, Zeng, Li, & Ertbjerg (2017) incubated myofibrils with either m-calpain, the proteasome or a lysosomal extract and found improved water-holding in parallel to degradation of myofibrillar proteins in and around the Z-disk. They hypothesized that degradation of cytoskeletal structure as indicated by desmin degradation and α-actinin release, allows water to flow from the space outside myofibrils to inside, thereby increases water-holding (Fig. 13).

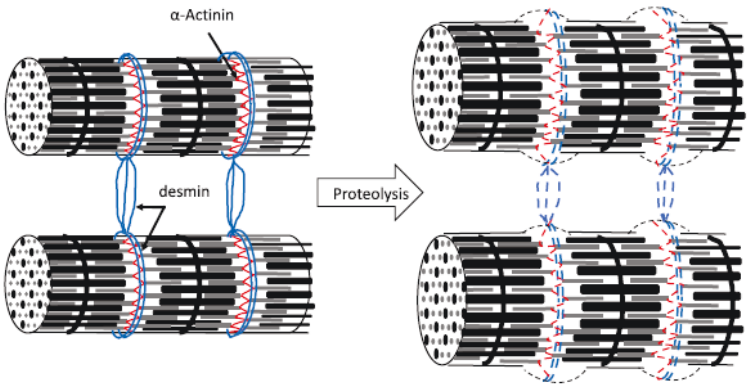

Fig. 13. Hypothesis showing structural changes of myofibrils as influenced by proteolytic degradation of cytoskeletal proteins. Zeng et al., 2017.

It is generally agreed that protein oxidation often leads to cross-linking in meat (Section 2.2.3.2). While proteolysis weakens the myofibrillar structure and improves water-holding, protein cross-linking would strengthen the structure and may reduce water-holding. Liu et al. (2009) proposed a model for the effect of protein oxidation on swelling of myofibrils in the presence of salt (Fig. 14). The disulfide cross-links were hypothesized to restrict salt-induced swelling of myofibrils. However, in myofibrillar protein gels, cross-linking is essential for gel structure and may have a different influence on water-holding.

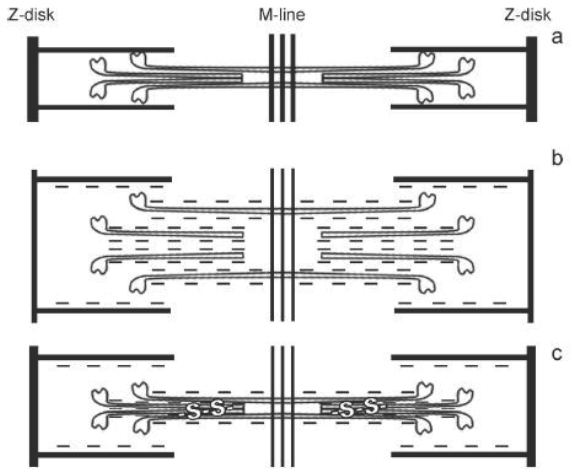

Fig. 14. Illustration of how disulfide cross-linkages would restrict the swelling of myofibrils. (a) a sarcomere with unswollen lattice space; (b) a sarcomere with swollen lattice space after salt irrigation; (c) a sarcomere with partially swollen lattice space which is restricted by disulfide cross-linkages after salt irrigation. Liu et al., 2009.

2.3.3. Effect of protein oxidation on premature browning of cooked meat

Meat color largely depends on the content and chemical state of its pigments, mainly the myoglobin. In MAP, oxygen promotes the oxygenation of myoglobin and therefore fresh red meat maintains bright red due to oxymyoglobin. Increased concentrations of oxygen generally increase red color stability of raw meat (Bartkowski, Dryden, & Marchello, 1982; Jakobsen & Bertelsen, 2000). However, high oxygen MAP often leads to premature browning in cooked meat (Clausen et al., 2009; Seyfert, Mancini, & Hunt, 2004; Suman et al., 2005; Study II).

In cooked meat, depending on end temperature, myoglobin mainly exist in denatured form. Denaturation of different forms of myoglobin eventually result in brown color (the cooked color) (Suman, & Joseph, 2013). However, denaturation temperatures of met-, oxy- and deoxymyoglobin are different and deoxymyoglobin was shown to be the most heat-resistant in terms of denaturation (Machlik, 1965; Hunt et al., 1999). Denaturation of myoglobin at lower temperatures leads to premature browning, a condition in which meat or meat products develop well done appearance (brown) while the safe internal temperature sufficient to kill pathogens has not been reached (Hague et al., 1994). For example, Boqvist, Fernström, Alsanius, & Lindqvist (2015) found that in hamburgers made from minced meat stored in 80%

oxygen, the survival of *Escherichia coli* O157:H7 was higher (as compared to atmospheric packaging) when visual color score was used to decide doneness. For consumers using the disappearance of red meat color to evaluate when the cooking is done, the premature browning phenomenon therefore poses a safety risk, especially in meats where microorganisms can be found not only on the surface (e.g. minced meat). The risk is even greater for consumers who prefer meat cooked to lower temperatures.

Premature browning is influenced by the susceptibility for heat-induced denaturation of different chemical status of myoglobin (deoxy-, oxy- and met-myoglobin). Denaturation of metmyoglobin results in the dull-brown ferrihemochrome, while denaturation of oxymyoglobin and deoxymyoglobin leads to pink-red ferrohemochrome, which may oxidize to ferrihemochrome (Suman & Joseph, 2013).

The chemical status affects the denaturation temperature of myoglobin. Hunt, Sørheim, & Slinde (1999) found that beef patties containing predominantly deoxymyoglobin showed typical red/pink to brown internal color during cooking from low to high temperature. Patties with predominantly oxymyoglobin and metmyoglobin were brown already at 55 ºC and became more brown at higher temperatures. They suggested that deoxymyoglobin does not denature easily at lower temperatures, and lack of heat-stable deoxymyoglobin induced premature browning. In agreement, Sepe et al. (2005) found that addition of food-grade reducing agents (sodium erythorbate, erythorbic acid, sodium ascorbate, ascorbic acid and ascorbyl palmitate) generally decreased the incidence of premature browning in ground beef, likely due to reduction of meat pigment to deoxymyoglobin.

The protein structure of myoglobin has an impact on thermal stability as well as on the autoxidation of oxymyoglobin and deoxymyoglobin to metmyoglobin (Suman & Joseph, 2013). Oxidative modifications likely alter the protein structure, which may consequently affect autoxidation and thermal stability of myoglobin. Kitahara, Matsuoka, Kobayashi, & Shikama (1990) confirmed that the autoxidation rate of myoglobin was accelerated by the presence of oxidizable cysteine residues.

Today, a large part of fresh meat in many countries is stored under high oxygen atmospheres (70% - 80% O_2) which promote lipid oxidation and protein oxidation. Oxidative stress may transfer to myoglobin and result in less heat-stable myoglobin and increase the chance of premature browning in cooked meats. There is recently a trend, however, to store meat in vacuum to avoid negative effects of oxygen.

2.4. Methods for investigation of protein oxidation in meat

As reviewed by Hawkins, Morgan, & Davies (2009), various methods can be applied to the quantification of protein oxidation in biological systems. Those methods are generally based on monitoring changes in parent amino acid residues (Cys, Met, His, Lys etc.), detection of radical and non-radical intermediates, or formation of products (e.g. formation of carbonyls, protein cross-linking). This section focus on generally used analytical methods in meat/muscle systems.

2.4.1. Carbonyl groups in proteins

A carbonyl group (C=O) is a functional group composed of a carbon double-bonded to an oxygen atom. Although the changes of carbonyl contents in biological system are not exclusively due to oxidative modification, determination of carbonyls has been used as a convenient tool for assessing protein oxidation.

Several chemicals can react with carbonyls and thus used for detection, including tritiated borohydride, 2,4-Dinitrophenylhydrazine (DNPH), fluorescein thiosemicarbazide, fluorescein amine – cyanoborohydride (Levine et al., 1990) and p-amino benzoic acid (Akagawa et al., 2006). DNPH is the most commonly used reagent in the determination of protein carbonyls. It is based on the reaction between carbonyl groups and DNPH (Fig. 15), which leads to formation of a hydrazone that can be detected spectrophotometrically at 370 nm or analyzed using antibodies against DNPH (Levine, Wehr, Williams, Stadtman, & Shacter, 2000).

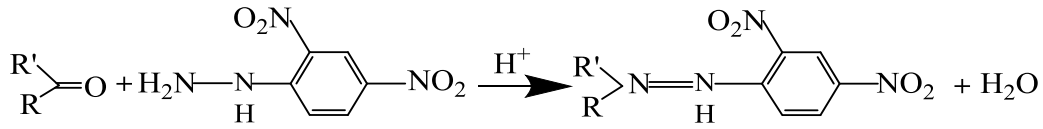

Fig. 15. Reactions of carbonyl group and DNPH.

Formation of protein carbonyls has been widely used as a maker for protein oxidation in meat (Estévez, 2011). The original method for carbonyl determination (Levine et al., 1990) has been adopted and modified to optimize the detection of carbonyls in muscle/meat proteins (Decker et al., 1993; Fagan, Sleczka, & Sohar, 1999; Soglia, Petracci, & Ertbjerg, 2016). Like many other tissues, the composition of meat is very complex and some of its components may interfere with the measurement of the actual

oxidation extent of the protein fraction. Therefore, the method for protein-bound carbonyl determination in meat usually involves protein separation steps such as precipitation of proteins with cold trichloroacetic acid (TCA) solution. However, oxidation of proteins may result in released carbonyls (such as formaldehyde, acetaldehyde, acetone, etc.) other than those bound to proteins (Headlam & Davies, 2004). The released carbonyls are not precipitated by TCA and thus lead to an underestimation of the carbonyl groups.

Another challenging aspect is to solubilize the oxidised proteins. Myofibrillar proteins and connective tissue proteins by nature are difficult to dissolve, and oxidation of those proteins results in even lower solubility. In study III, it was found that even with high concentration of urea (8 M) or guanidine hydrochloride (6 M), only part of the highly oxidized myofibrillar proteins can be solubilized.

Based on the traditional DNPH method for carbonyl detection, Soglia et al. (2016) introduced treatments including addition of SDS, heating, and ultra-sonication for TCA precipitated protein before derivatization with DNPH, and they found those additional steps overall resulted in two to four fold more carbonyls measured in meat from different species as well as in different protein fractions. The increased labelling of protein carbonyls with DNPH was likely due to both increased solubility of proteins and more unfolding of the protein structure.

The traditional DNPH-based method measures total protein carbonyls which are formed by diverse and unspecific pathways (Estévez, 2011). Estévez, Ollilainen, & Heinonen (2009) suggested that LC-MS analysis of specific carbonyls (e.g., AAS and GGS) would provide more precise information about mechanisms involved in food protein oxidation. Determination of AAS and GGS has been successfully applied in meat systems and formation pathways of AAS from lysine and GGS from arginine or proline were proposed (Estévez, 2011). In addition to the abovementioned 3 amino acid residues (lysine, arginine and proline), some other amino acids can also be oxidized and form specific carbonyls. One good example is histidine-derived carbonyl 2-oxo-histidine, which has been detected in many proteins subjected to in vitro oxidation (Uchida, 2003). In Study III a decrease of histidine and an increase in total carbonyl content of myofibrillar proteins were found due to oxidation, suggesting that histidine-derived specific carbonyls can be served as a good biomarker for protein oxidation in meat.

2.4.2. Free thiol groups in proteins

A thiol (R-SH) is a carbon-bonded sulfhydryl group and it is highly susceptible to oxidation. Loss of free thiol groups is widely used as an indicator of protein oxidation.

The predominant method for detection of thiol groups is a spectrophotometric method based on 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman, 1959). A free thiol reacts with DTNB and forms 2-nitro-5-thiolbenzoic acid (TNB) anion (Fig. 16), which absorbs strongly at 412 nm.

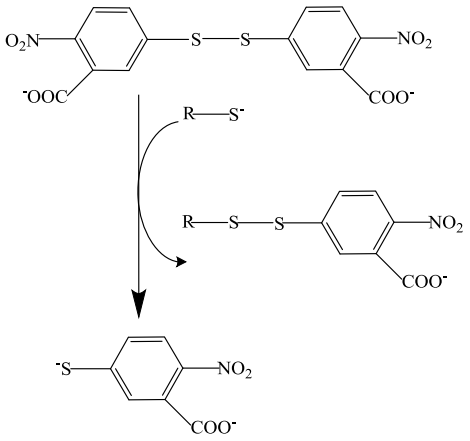

Fig. 16. Reactions of thiol group and DTNB.

Different values for extinction coefficient of TNB anion at 412 nm, ranging from 13,600 M^{-1} cm^{-1} to 14,150 M^{-1} cm^{-1}, were reported in the literature (Ellman, 1959; Riddles, Blakeley, & Zerner, 1979, 1983; Riener, Kada, & Gruber, 2002). For proteins with more free thiol groups, inaccurate use of extinction coefficient would lead to less precise quantification. According to Riddles et al. (1979), the purified TNB has a value of 14,150 M^{-1} cm^{-1} and this value varies depending on factors like pH and buffer system. As an alternative way to quantify thiol groups, a standard curve can be prepared using L-cysteine (Møller, Stapelfeldt, & Skibsted, 1998).

Only the thiolate form reacts with DTNB (Riddles et al., 1983), therefore the reaction depends on pH and the pK_a of thiol group. For isolated cysteine, the thiol group has a pK_a value around 8.7 which is similar to pH of generally used reaction buffer. However, pK_a values for thiol groups in proteins can be

greatly affected by interaction with neighbouring amino acids. Denaturing buffer can be used to unfold proteins and thereby expose the thiol groups.

Alternative chemicals/methods exist for analyzing thiol groups, such as aromatic disulfide reagents, fluorescent thiol reagents, thiol reagents that significantly increase molecular weight and this has been thoroughly discussed in a previous review (Hansen & Winther, 2009).

The quantification result of thiol groups is generally based on protein amount. If absorbance at 280 nm is to be used for protein determination, it is recommended to use a conversion factor of 1 mg/mL protein equals to 1 unit absorbance. For a protein mixture, this conversion is more accurate than preparing a standard curve from bovine serum albumin (BSA) (1 mg / mL BSA corresponds to about 0.76 absorbance at 280 nm). As the thiol group is very reactive, its loss in meat/meat products may due to reactions other than oxidation, such as interactions with polyphenols (Jongberg, Skov, Tørngren, Skibsted, & Lund, 2011).

2.4.3. Protein cross-links

Upon oxidation, muscle proteins can form various cross-linked products and disulfide is one of the main cross-linking type found in meat (Lund et al., 2011). Non-disulfide type of cross-linking, including dityrosine and carbonyl-involved cross-links, occurs in meat systems as well (section 2.1.3).

Analysis of disulfides is based on the analysis of thiol groups. The detection of disulfides usually involves a series of steps: thiol blocking, disulfide reduction, and detection of newly formed thiols (Hansen & Winther, 2009).

Protein cross-linking via covalent bonds lead to an increased molecular weight and therefore cross-linked proteins migrates to a higher position in a SDS-PAGE gel. By running SDS-PAGE with and without reducing agent (which reduce the disulfide bond), information about the cross-linking type can be obtained.

Disulfide cross-linking can be both intra- and inter-molecular. Diagonal PAGE has been used as a tool to distinguish the two types of protein cross-linking (Winger, Taylor, Heazlewood, Day, & Millar, 2007). The diagonal PAGE is a 2D gel which separates proteins based on molecular weight first under non-reducing condition and then under reducing condition (Fig. 17). This technique has been used to study protein cross-linking in high oxygen packaged beef (Kim et al., 2010; Moczkowska et al., 2017).

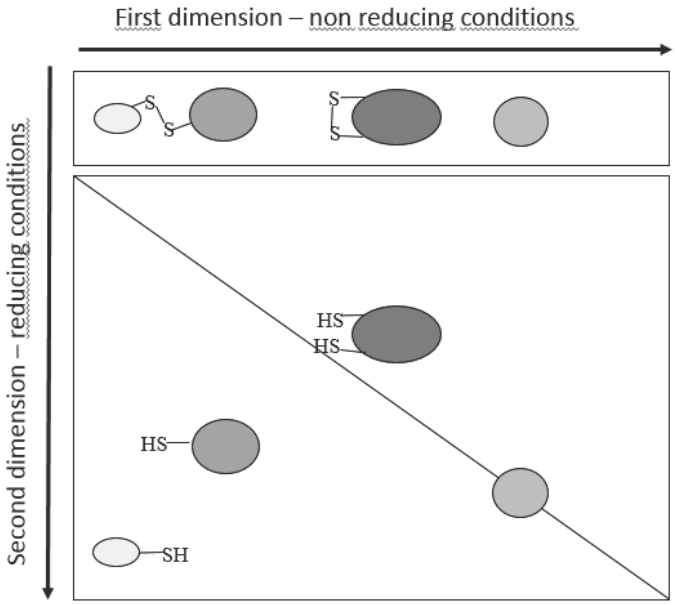

Fig. 17. An illustration of the principle of 2D diagonal SDS-PAGE for the identification of intra- and intermolecular disulfide bonds. Proteins with a lack of reducible disulfide bonds run in the second dimension on the diagonal; proteins with intermolecular disulfide bonds migrate below the diagonal; proteins with intramolecular disulfide bonds migrate above the diagonal.

Some of the myofibrillar proteins (e.g. titin and nebulin) have a very large molecular weight and low solubility. Inter-molecular protein cross-linking leads to even larger and less soluble molecules, thereby making the analysis of oxidized proteins by gel electrophoresis rather difficult. Warren, Krzesinski, & Greaser (2003) developed a vertical agarose gel electrophoresis system that allows separation of titin and other high molecular weight products. As for the solubility enhancement, detergents such as SDS and/or a high concentration of urea / thiourea can be used.

Particle size as determined by laser diffraction has been previously used as a fast and simple method to evaluate myofibril fragmentation in meat (Karumendu, van de Ven, Kerr, Lanza, & Hopkins, 2009; Lametsch, Knudsen, Ertbjerg, Oksbjerg, & Therkildsen, 2007). In a laser diffraction measurement, a laser beam passes through a dispersed particulate sample and scatters, and the angles of scattered light depend on the particle size (Fig. 18A). The particle size is reported as a volume equivalent sphere

diameter (Fig. 18B). Sample preparation has a great effect on particle size distribution, a high homogenization speed is recommended (Karumendu et al., 2009)

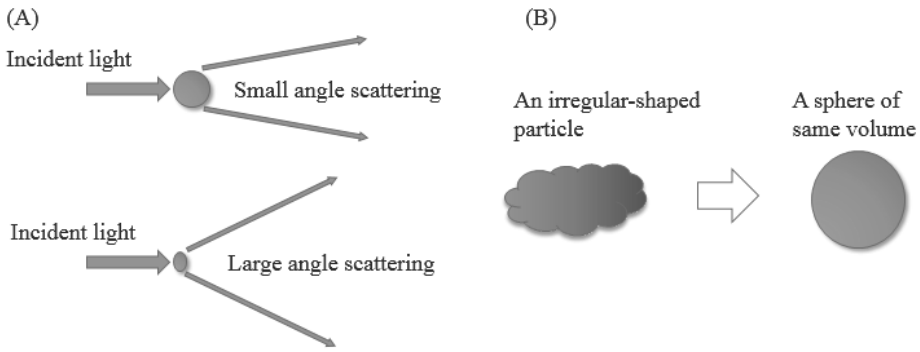

Fig. 18. Scattering of light from small and large particles (A) and an illustration of volume-equivalent sphere (B).

Intermolecular protein cross-linking is expected to increase the size of particles in meat homogenates. In this thesis, particle size was found to be a useful indicator of oxidation-induced protein cross-linking. In general, particle size increases with increasing oxygen concentrations in MAP (study II) or increasing oxidant (HClO) concentrations (study III).

3. Objectives

The aims of the thesis were to investigate oxidation-induced changes at the level of proteins and amino acids and to explore how these modifications will affect meat quality traits such as color, texture and water-holding. To achieve this, experiments were performed at different scales (intact meat pieces, minced meat and myofibrils) and different conditions were used to induce oxidation (MAP with oxygen, incubation with HClO). The specific aims were:

- To investigate the effect of MAP with different oxygen concentrations on protein oxidation, proteolysis, and shear force of porcine LD muscle, and explore the mechanism of oxidation-induced textural changes in fresh meat (Study I)

- To investigate the effect of MAP with different oxygen concentrations on lipid and protein oxidation in minced beef and their relationship to premature browning and hardness of patties after cooking to different end-point temperatures (Study II)

- To investigate the effect of HClO-mediated oxidation on the net charges of myofibrillar proteins and to provide insight into the mechanism of oxidation-induced changes in water-holding and aggregation (Study III)

4. Materials and methods

A brief summary of the materials and methods used in the present thesis is described in this section, and more details can be found in the attached publications (study I, II & III).

4.1. Muscles

In meat science, a major muscle studied in both pork and beef is the LTL. Porcine LTL muscles were used in study I & III. Bovine triceps brachii muscles were used in paper II as this study focus on color (premature browning) which mainly has been reported as a problem in beef, and also beef generally has higher concentration of myoglobin than pork. The pigs belonged to the cross Norwegian Landrace × Swedish Yorkshire × Danish Landrace and were slaughtered at a conventional slaughterhouse in Finland. Beef shoulders from Ayrshire bulls were purchased from Heikin Liha Oy (Helsinki, Finland). LTL muscles were excised at 24 hours postmortem, vacuum packaged and transported refrigerated to the lab (study I & III). Each muscle was trimmed of external fat and connective tissue (study I, II & III).

4.2. Oxidation conditions

Muscles were sliced into pieces (study I) or minced (study II) and then these samples were allocated for MAP with different oxygen concentrations (0, 20, 40, 60 and 80%) and stored at chilled temperature for 14 days (study I) or 6 days (study II), receiving light for 12 h every day from tubular fluorescent lamps (study I & II). In study III, myofibrils were extracted with MES buffer (100 mM KCl, 50 mM MES (2-(N-Morpholino) ethanesulfonic acid hydrate), 2 mM $MgCl_2$, 2 mM EGTA (ethylene glycol tetraacetic acid), pH 5.5) and incubated with different concentrations of HClO (0, 1, 5 and 10 mM) overnight at 5 ºC. Preliminary experiments showed that HClO was more efficient in introducing protein carbonyls than Fenton reaction system. To achieve similar extend of protein carbonylation, the latter system thereby involves incubation for longer time or at higher temperatures. The concentrations of HClO to be used were pre-tested to ensure that the pH of the system in the experiments was minimally affected.

4.3. Physical analysis

Allo-Kramer shear force (study I)

Determination of Allo-Kramer shear force was conducted as described by Liu, Ruusunen, Puolanne, & Ertbjerg (2014). Meat pieces of 20 × 20 × 6 mm (muscle fibre along the 20 mm direction) were placed

in an Allo-Kramer shear cell and cut across the fibre direction. The results were expressed as peak force divided by the weight of the sample, N/g.

Hardness of patties (study II)

Patties of 80 g (diameter about 10 cm, thickness about 12 mm) were prepared from minced beef by a household hamburger maker. Beef patties were cooked at different temperature (55, 60, 65, 70 ºC) for 1 h in a water-bath. Cooked beef patties were cut into small cubes of 2 cm × 2 cm × patty height and compressed to 60% with a probe of 6 mm in diameter using a TA.XT plus texture analyzer. Each sample was compressed once from the middle in the orientation of patty height. The peak force (kg) was recorded as hardness.

Purge loss (study I) and cook loss (study I & II)

Purge loss was measured by taking the weight of sample before and after storage and expressed as percentage loss of initial weight. Cook loss was determined as the percentage loss of initial weight after cooking.

WHC as determined by centrifugation (study III)

Water-holding of myofibrils was determined by centrifugation method. Myofibrils were suspended in 50 mM MES (2-(N-Morpholino) ethanesulfonic acid hydrate) buffer (pH 5.5) and centrifuged at 2,400 g for 10 min followed by discard of the supernatant. Water-holding was defined as the amount of water held in the pellet per gram myofibrillar protein.

Particle size (study II & III)

Particle size of meat homogenates (study II) and myofibril suspensions (study III) were analyzed by a Mastersizer 3000 (Malvern Instruments Ltd., Malvern, UK) which is based on light scattering. The refractive index was set to 1.46 and the absorption coefficient to 0.01, and the particles were considered as non-spherical. Volume weighted distribution was used.

$D(v,0.1)$ – the size of the particle for which 10% of the sample is below this size;

$D(v,0.5)$ – the size of the particle for which 50% of the sample is below this size;

$D(v,0.9)$ – the size of the particle for which 90% of the sample is below this size;

$D(3,2)$ – the surface area moment mean diameter, $D(3,2) = \sum n_i d_i^3 / \sum n_i d_i^2$, where n_i is the number of particles with diameter d_i and was calculated from the size distribution;

$D(4,3)$ – the volume moment mean diameter, $D(4,3) = \sum n_i d_i^4 / \sum n_i d_i^3$, where n_i is the number of particles with diameter d_i and was calculated from the size distribution.

Color (study II)

Color was measured with a Minolta Chroma meter CR-400 (Minolta Camera Co. Ltd., Osaka, Japan) set at D65 illuminant at room temperature. The diameter of measuring aperture was 8 mm. The instrument was calibrated using a white tile (C: Y = 93.6, x = 0.3130, y = 0.3193). The L* (lightness), a* (redness) and b* (yellowness) values were recorded.

4.4. Biochemical analysis

Protein oxidation (study I, II & III)

Protein oxidation was measured as loss of free thiols (study I, II and III) and formation of carbonyl groups (study III). The free thiol content was determined using a DTNB-based (DTNB, 5,5'-Dithiobis(2-nitrobenzoic acid)) method (Ellman, 1959) with modifications as described in study I, II and III. The carbonyl content was determined according to Soglia et al. (2016).

Lipid oxidation TBARS (study I & II)

TBARS were measured according to the method of Salih, Smith, Price, & Dawson (1987), with some modifications by Utrera, Morcuende, & Estévez (2014). Briefly, muscle sample (5.0 g) was homogenized with 15 mL trichloroacetic acid (5%, w/v) and 0.5 mL butylated hydroxytoluene (4.2% in ethanol, w/v) and filtered with filter paper. An aliquot of 2 mL filtrate was mixed with 2 mL thiobarbituric acid (0.02 M) in test tube and incubated at 100 ºC for 40 min. Absorbance was read at 532 nm after cooling. A standard curve was prepared using 1,1,3,3-tetraethoxypropane (hydrolyses to give MDA). TBARS content was expressed as mg malondialdehyde / kg meat.

SDS-PAGE (study I & II)

Diluted filtrates prepared for measurement of thiol groups were used for SDS-PAGE analysis. Protein content of the filtrates was determined by RC DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). NuPAGE (Invitrogen, CA, USA) Novex 12% Bis-Tris gels (study I) and 3-8% Tris-Acetate gels (study I and II) were used.

IEF gel (study III)

Myofibrillar proteins were extracted at room temperature overnight with extraction solution (8 M urea, 2 M thiourea, 1% CHAPS). After extraction, the mixture was centrifuged and mixed (1: 1) with 2x sample buffer (8 M urea, 2 M thiourea, 1% CHAPS, 80 mM lysine, 15% glycerol) and centrifuged. The resultant supernatant was loaded onto Novex pH 3 – 7 Isoelectric Focusing gels (Thermo Fisher Scientific). After IEF separation, the gel was washed for 5 min in deionized water and then fixed in 12% TCA for 30 min. After fixation, the gel was washed 3 x 10 min with deionized water and then stained in Coomassie Brilliant Blue R250 solution.

Western-blot against desmin (study I) and MHC (study I & III)

Prior to blotting, the IEF gel was treated according to Anderson & Peck (2014). Briefly, the gel was first washed for 5 min in deionized water and fixed in 12% TCA at 5 ºC overnight. Then the gel was washed 3 x 10 min followed by incubation in resolubilization buffer (7 M urea, 2 M thiourea, 5 mM dithiothreitol) for 10 min and deionized water for 5 min. Finally, the gel was incubated in SDS equilibration buffer (0.37 M Tris-HCl, pH 8.8, 0.1% SDS) for 3 x 5 min.

Proteins in SDS-PAGE gels and IEF gels were transferred at 30 V for 1 h onto Immobilon-FL Transfer Membranes (Millipore, Bedford, MA). The transfer buffer was prepared from NuPAGE Transfer Buffer (20×) and 10% methanol was added for desmin analysis (study I), while 5% methanol and 0.05% Tween-20 were added for MHC analysis (study I and III). Membranes were probed and quantified according to Liu et al. (2014). Desmin was detected by mouse monoclonal anti-desmin antibody clone DE-R-11 (Santa Cruz, CA, USA) at 1:5000 and MHC was detected by mouse monoclonal anti-myosin (Skeletal, Fast) antibody clone MY-32 (Sigma-Aldrich, Saint Louis, US) at 1:40000 (study I) or 1:5000 (study III).

Calpain activity (Study I)

Frozen samples were finely chopped and 1.5 g meat was homogenized at 13500 rpm for 30 s in cold extraction buffer (50 mM Tris, 5 mM EDTA, 10 mM monothioglycerol, pH 8.0). The homogenate was centrifuged at 15,000 g for 30 min at 4 ºC. An aliquot of the supernatant was mixed with glycerol to final concentration 30% and stored at -60 ºC. The activity of calpain was determined by casein zymography as described (Pomponio et al., 2008).

Surface hydrophobicity (study III)

Surface hydrophobicity of the myofibril in MES buffer was indicated by protein-bound bromophenol blue which binds to hydrophobic surface areas of folded protein. Briefly, a standardized amount of myofibrils were incubated with bromophenol blue, and following a centrifugation the amount of dye left in the supernatant was used to calculate the protein-bound dye. The measurement was based on Chelh, Gatellier, & Santé-Lhoutellier (2006) with modifications by Liu et al. (2016).

Protein identification (study III)

In-gel digestion followed by peptide identification with nLC-MS/MS was used for protein identification.

Peptide samples were measured by nLC-MS/MS with a Proxeon EASY nLC and a LTQ-Orbitrap XL mass spectrometer as previously described (Lu et al., 2011). MaxQuant 1.5.2.8 was used for protein identification and quantitation of the LC–MS data (Cox & Mann, 2008). The "Specific Trypsin/P" Digestion mode was used with maximally 2 missed cleavages and further default settings for the Andromeda search engine (Cox et al., 2011).

Amino acid analysis (study III)

Myofibrillar proteins were digested in HCl and the amino acids were derivatized with Waters (Milford, MA, USA) AccQ•Tag Ultra reagent and analyzed by UPLC on Acquity system (Waters) equipped with a Waters BEH C18 column (100 mm * 2.1 mm, particle size 1.7 µm). Details about sample preparation, UPLC running condition and peak identification / quantification can be found in section 2.7, study III.

Statistical analysis (Study I, II, III)

Analysis of variance (ANOVA) was done by IBM SPSS Statistics 24 software. Tukey HSD (honest significant difference) test was used to find significant differences at a level of $P < 0.05$.

5. Summary of results

5.1. Effect of oxidative conditions on protein oxidation and lipid oxidation

Protein oxidation

Loss of free thiol groups was used as a general marker for protein oxidation. Free thiols of meat proteins decreased after cold storage in MAP (Fig. 3, study I; Fig. 4, study II). The free thiol content in porcine LD muscle at day 0 was 61 nmol/mg protein and up to 17% was lost after storage for 14 days; while in bovine TB muscle the free thiol content was 50 nmol/mg protein at day 0 and up to 20% was lost after storage for 6 days. Storage in MAP with oxygen resulted in lower free thiol groups as compared to 0% oxygen MAP, and no difference in free thiol content was found between varying oxygen concentrations (20% - 80%). In HClO-mediated oxidation of porcine myofibrils, oxidation led to a decrease of free thiol groups from 98 nmol/mg protein in the non-oxidized control to 55 nmol/mg protein in 10 mM HClO oxidized samples (Fig. 1, study III). Formation of protein carbonyls was used as another general marker for protein oxidation in HClO-mediated oxidation of isolated myofibrils (Fig. 2, study III). Carbonyls increased from 2.0 nmol/mg protein in control groups to 11.0 nmol/mg protein at 10 mM HClO.

The oxidation-induced protein cross-linking was used as a specific marker of protein oxidation. Electrophoresis revealed a high molecular weight protein band (migrated at a position in the gel higher than 460 kDa molecular weight standard, especially in samples that stored in high oxygen MAP (Fig. 4, study I; Fig. 5, study II, protein standard lane not shown). Western blot using a monoclonal antibody against MHC confirmed that the high molecular weight band contained MHC and the band was referred to as cross-linked myosin heavy chain (CL-MHC) (Fig. 4, study I). When reduced with dithiothreitol (DTT), CL-MHC band was less noticeable and less protein remained on top of the gel, indicating that disulfide-type cross-linking was involved. The relative band intensity of MHC and CL-MHC after aging was calculated as the ratio to the band intensity of MHC at day 0 and presented in Fig. 5, study I. Oxygen significantly affected the intensity of the MHC band ($P = 0.01$) and CL-MHC band ($P < 0.01$). The intensity of CL-MHC was greater in 80% oxygen packaged samples compared to 0 and 20% oxygen.

Lipid oxidation

Lipid oxidation was measured as TBARS and oxygen significantly promoted ($P < 0.01$) lipid oxidation in meat (Fig. 2, study I; Fig. 3, study II). Storage in 0% oxygen MAP did not increase lipid oxidation

compared to day 0. However, storage in oxygen-containing MAP greatly enhanced the formation of lipid oxidation products. After storage, the TBARS value in porcine LD muscle was only 0.03 mg MDA / kg meat in 0% oxygen MAP but 80% oxygen led to a ten-fold increase to 0.34 mg MDA / kg meat. In minced beef muscle, the TBARS value was around 0.17 mg MDA / kg meat in 0% oxygen and increased to 1.24 mg MDA / kg meat in 80% oxygen. Unlike free thiol groups, lipid oxidation generally increased with increasing oxygen concentrations (20-80%) in MAP.

5.2. Consequences of oxidative modifications on meat proteins

Increased particle size

Particle size distribution was used to evaluate the structural changes of meat proteins. Generally, D(3,2) of particles in raw meat homogenates were smaller after storage for 6 days except for samples in 60% oxygen (Table 2, study II), indicating a tenderization effect of minced beef due to aging. In stored samples, D(3,2) increased with increasing oxygen concentrations up to 60% (Table 2, study II). A similar effect of oxygen on the particle size of cooked meat was also observed (Table 3, study II): increasing oxygen showed a clear trend of increasing particle size. Cooking temperatures from 55 to 65 ºC generally increased particle size and cooking led to an increase of about 2-4 fold. For isolated myofibrils oxidized with HClO, all the reported statistical parameters of particle size (D(v,0.1), D(v,0.5), D(v,0.9), D(3,2), and D(4,3)) increased ($P < 0.001$) with increasing concentration of HClO (Table 1, study III). The increased particle size was supported by microscope images of the oxidized myofibrils (Fig. 19)

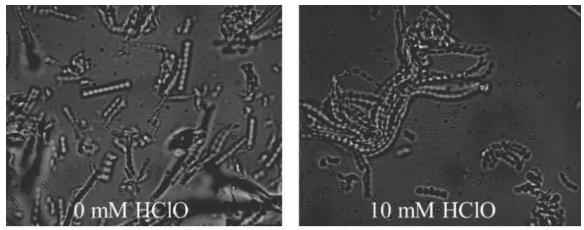

Fig. 19. Light microscope images taken at magnification ×1000, of extracted myofibrils after incubation without (0 mM) or with HClO (10 mM).

These results of particle size clearly indicated that oxidation promoted cross-linking and aggregation of meat proteins. Surface hydrophobicity was also measured for oxidized myofibrillar proteins (Fig. 20) and it did not explain the intense aggregation as no significant difference was found between 10 mM

HClO group and the control. Other factors such as protein cross-linking are therefore more likely to have contributed to the aggregation.

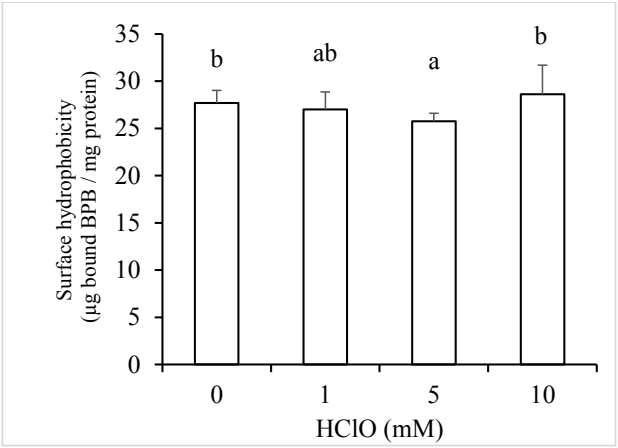

Fig. 20. Surface hydrophobicity of myofibrillar proteins after incubation with different concentrations of HClO.

Shift of pI

Myofibrillar proteins were extracted after oxidation and then separated on IEF gels to determine their pI (Fig. 4, study III). Oxidation caused a general shift of pI of proteins in boxes 1-4 to a slightly more acidic area. Proteins in boxes 1 and 2 contained myosin heavy chain as identified by western blot (Fig. 4B, study III), while the box 3 mainly contained tropomyosin, actin and myosin light chain and box 4 contained actin and myosin light chain as identified by mass spectrometry (Fig. 4C, study III). The lower pI after oxidation suggests that the amount of net negative charges of myofibrillar protein increase. This effect is also observed in other oxidation systems as a shift of pI to more acidic values following oxidation was also observed in a preliminary test using Fenton reaction (data not shown).

By the IEF technique it was only possible to study the solubilized myofibrillar proteins, however, a fraction of very strongly oxidized myofibrils remained insoluble. To study the insoluble part of myofibrillar proteins, the oxidized myofibrils were hydrolyzed with HCl and subjected to amino acid analysis. The highest oxidation level (10 mM HClO) led to a 14% decrease ($P = 0.03$) of histidine when compared to the non-oxidized control. No other analyzed amino acids were found to be significantly changed due to oxidation (Table 2, study III). The decrease of positively charged histidine residues in oxidized myofibrils would be expected to lead to an increased amount of net negative charges, in

agreement with the observation that pI of the oxidized myofibrillar proteins shifted to lower values in IEF gel which also indicated an increased net negative charges.

Proteolysis not affected

Degradation of desmin was used as an indicator of myofibrillar protein proteolysis. After storage in MAP, 70% - 80% desmin was degraded into lower molecular weight products and no significant effect of oxygen on desmin degradation was found (Fig. 6 & Fig. 7, study I).

The disappearance of μ-calpain band in casein zymography gels indicated that μ-calpain is not inactivated by the oxygen in MAP (Fig. 21), if it has been inactivated by the oxidative stress, the activity could be restored when reducing conditions were used in casein zymography. The occurrence of bands at location 4 indicated the autolysis of m-calpain.

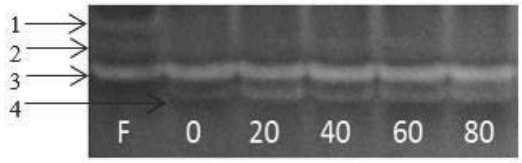

Fig. 21. Representative Zymogram showing calpain activity in porcine *longissimus thoracis et lumborum* (LTL) muscle stored in modified atmosphere with different oxygen concentration (0, 20, 40, 60 and 80%) at 5 ºC for 14 days. F represents 1 day postmortem samples. 1, μ-calpain; 2, autolyzed μ-calpain; 3, m-calpain; 4, autolyzed m-calpain

5.3. Effect of oxidative conditions on meat quality traits

Texture

Generally, oxygen in MAP led to a significant increase ($P < 0.01$) of Allo-kramer shear force of porcine LD muscle (Fig. 1, study I) and hardness of cooked beef patties (Table 3, study II), demonstrating a meat-toughening effect. At relative lower oxygen concentrations (20%, 40%), shear force was higher than at 0% oxygen, and increased further at higher oxygen concentrations (60%, 80%). At lower cooking temperatures (55 ºC, 60 ºC), beef patties made from minced meat packaged under oxygen (20-80%) had larger hardness values than those from 0% oxygen ($P < 0.05$); however, there were no clear effects of

oxygen on hardness at higher cooking temperatures. In study II, there was a significant correlation (r = 0.8, P < 0.01) between particle size and hardness of cooked patties after 6 days storage in MAP (Fig. 2, study II).

Color

Packaging did not affect L*, a* and b* values of porcine LD muscle after storage 14 days in MAP (Table 5).

Table 5. Effect of oxygen concentrations in MAP on the color of chilled stored (14 days) porcine LD muscle.

Color	**Day 0**	**Oxygen concentration (%), day 14**					**SEM**	**Main effects[A]**
		0	20	40	60	80		**(*P* values)**
L*	53.3	57.8	58.1	58.0	57.4	58.0	0.5	NS (0.99)
a*	6.4	6.7	5.3	5.6	5.5	4.2	0.3	NS (0.13)
b*	3.8	7.7	8.4	8.2	8.2	7.9	0.3	NS (0.87)

A: Main effects of oxygen concentration at day 14. NS: not significant (P > 0.05).
SEM: standard error of the mean.

In minced beef, however, oxygen concentration had a significant main effect (P < 0.05) on L*, a* and b* values. High oxygen MAP generally had larger values of a* and b* as compared to 0% oxygen (Table 2, study II). Packaging in MAP with oxygen also affected the internal color of cooked beef patties (Fig. 1 and Table 3, study II) and resulted in premature browning. Patties that were made on day 0 were more red and less brown than made from stored meat. The cooked appearance (brown color) generally increased with increasing cooking temperature and oxygen concentration in MAP for day 6 samples. It can be seen (Fig. 1, study II) that a combination of high oxygen and low cooking temperature can lead to a comparable or even more brown internal color than patties in lower oxygen MAP cooked at higher temperatures. Therefore, packaging with oxygen was able to cause premature browning in minced beef and premature browning was observed already at 20% oxygen, a relative low concentration, which is similar to the amount in atmosphere.

Water-holding

Oxygen in MAP did not affect the purge loss or cook loss of porcine LD muscle (Table 6).

Table 6. Effect of oxygen concentrations in MAP on purge loss and cook loss of chilled stored (14 days) porcine LD muscle.

Oxygen concentrations (%)	**Purge loss (%)**	**Cook loss (%)**
0	10.4	17.6
20	10.8	16.1
40	10.8	20.0
60	10.8	19.4
80	10.8	21.2
SEM	0.3	1.2
Main effect of oxygen	NS	NS

SEM, standard error of the mean. NS, not significant ($P > 0.05$)

Cook loss of beef was significantly ($P < 0.01$) affected by oxygen concentration in MAP and was lower in patties made from high oxygen packaged meat (Table 7). Increasing cooking temperature increased cook loss as expected.

Table 7. Cook loss (%) of patties made from minced bovine *triceps brachii* muscle stored in MAP with different oxygen concentrations.

Temperature (°C)	**Oxygen concentrations (%)**					**SEM**	**Main effects[A]**		
	0	20	40	60	80		O_2	T	O_2 x T
55	15.5	15.4	14.5	14.5	14.5	0.4	**	***	NS
60	20.0	20.0	19.5	19.7	18.9				
65	24.6	23.2	22.5	22.8	22.6				
70	28.1	27.9	27.7	27.1	26.7				

[A]Main effects of oxygen (O_2), cooking temperature (T) and their interaction (O_2 x T) at day 6. Different significance levels are indicated as: ** ($P < 0.01$), *** ($P < 0.001$), NS (not significant).

SEM: standard error of the mean.

When myofibrils are used, water-holding can be evaluated by the amount of water kept by the myofibril pellet after centrifugation. In study III, oxidation with HClO increased ($P < 0.001$) the water-holding. Oxidation with 10 mM HClO increased water-holding from 6.6 g H_2O / g protein in the non-oxidized control to 7.3 g H_2O / g protein, about 11% more water was bound (Fig. 5, study III).

6. General discussion

6.1. Effect of oxidative conditions on the oxidation of proteins and lipids

Many studies have investigated effects of high oxygen MAP and vacuum packaging on protein oxidation and meat quality (section 2.3). In this thesis, the effect of oxygen concentration in MAP was studied by varying oxygen as the only variable (balanced by the inert gas N_2). Therefore, it was the oxygen in MAP that generally led to a loss of free thiols (Fig. 3, study I; Fig. 4, study II) and formation of TBARS (Fig. 2, study I; Fig. 3, study II). That the oxygen in MAP is able to induce protein oxidation or lipid oxidation are supported by many previous studies (Clausen et al., 2009; Kim et al., 2010; Lund et al., 2007; Spanos, Tørngren, Christensen, & Baron, 2016; Zakrys-Waliwander et al., 2012).

In Study III, hypochlorous acid (HClO) was used as the oxidant of myofibrils. HClO is produced *in vivo* and it is a strong oxidant capable to mediate protein oxidation (Hawkins, Pattison, & Davies, 2003). HClO has been previously used to induce protein oxidation in meat (Soglia et al., 2016) and it may be seen as an alternative to the widely used Fenton reaction. In study III, the HClO model oxidation system was able to oxidize proteins as evidenced by loss of free thiols (Fig. 1, study III) and formation of carbonyls (Fig. 2, study III). Histidine was found to decrease with oxidation (Table 2, study III), and the decrease was at the same magnitude as the formation of carbonyls. Lysine and arginine can form specific protein carbonyls and those specific carbonyls have been used as protein oxidation marker in meat (Estévez, 2011), but oxidation with HClO did not affect those residues. Therefore, oxidation of histidine alone was likely to give rise to the carbonyls.

Protein cross-linking, as indicated by the formation of cross-linked MHC, was more profound in higher oxygen concentrations (Fig. 4, study I). The loss of free thiols (Fig. 3, study I) and formation of lipid oxidation products (Fig. 2, study I) in high oxygen compared to 0% oxygen may contribute to disulfide cross-linking and MDA-mediated protein cross-linking, respectively. In agreement, formation of TBARS (Fig. 3, study II) and loss of free thiols (Fig. 4, study II), in parallel with formation of protein cross-links (Fig. 5, study II) were also greater in high oxygen packaged minced beef than 0% oxygen. In study II, the formed cross-links (CL-MHC and those that stayed on top of the gel) were largely, but not totally reduced by DTT (Fig. 5, study II), proved that the disulfide-type of cross-links and other types of cross-links were involved. Cross-linking were also supported by the microscopic images showing a big cluster of myofibrils in oxidized samples while small myofibril fragments dominate in the non-oxidized samples (Fig. 19), and also by greater particle size of HClO-oxidized myofibrils (Fig. 3 & Table 1, study III) and

high oxygen packaged minced meat (Table 2, study II). The loss of positively charged histidine (Table 2, study III) may have contributed to protein aggregation and therefore a larger particle size.

Theoretically, oxidative reactions could be transferred between lipids and proteins as suggested in Fig. 8, study I. Increased TBARS with increasing oxygen concentration in MAP (Fig. 2, study I; Fig. 3, study II) likely served as a pool of reactive species, such as MDA which may react with proteins. This is supported by Burcham, & Kuhan (1996) who showed that incubation with MDA introduced carbonyl groups into BSA. In agreement, Zhang, Xiao, Lee, & Ahn (2010) found that consumption of oxidized oil was related to higher protein carbonyl content in breast meat of broiler chickens. Liu (2017) investigated the effects of saturation level of added lipids on protein oxidation in minced pork, and the results showed that less saturated group had higher oxidative stability in relation to both lipid and protein oxidation. This is in contrast to general belief and may be caused by the higher amount of antioxidant (vitamin E) in less saturated lipid fractions. In study I & II, there was no difference in free thiols among groups in 20% – 80% oxygen MAP. This may be explained by that thiol groups can be oxidized into sulfenic acid resulting a loss of free thiols, but sulfenic acid can be further oxidized to sulfinic and sulfonic acid without changes in free thiol content (Ross, Foloppe, & Messens, 2013)

6.2. Oxidation-induced meat quality changes

6.2.1. Oxidation-induced meat toughening

From Table 1 & 2, it is clear that many studies, including study I & study II in current thesis, agree that oxidative conditions (MAP with oxygen, irradiation) lead to tougher meat, although some observed no difference in shear force or sensory tenderness in meat. One reason for the lack of effect could be that the sample size was too large so that oxygen did not penetrate to the inner part where shear force was measured (Lindahl et al., 2010). Two mechanisms have been proposed: toughening through formation of protein cross-links (Section 2.3.1.1) and less tenderization through reduced proteolysis (Section 2.3.1.2).

It is generally believed that protein cross-linking leads to meat toughening and the formation of protein cross-links in meat systems has been discussed in section 2.2.3.2. Protein cross-linking (Fig. 4, study I; Fig. 5, study II) was observed together with increasing shear force at higher oxygen concentration in MAP (Fig.1, study I) and increased hardness of cooked patties (Fig. 2, study II). In study II & III, protein oxidation also led to larger particle size which reflects on protein cross-linking. Therefore, this thesis

supports that cross-linking contributes to tougher meat texture. Previously, particle size has been used to reflect on proteolysis in meat. Lametsch et al. (2007) found particle size to be positively correlated with Warner-Bratzler shear force of porcine LD muscle and day 8 samples exhibited smaller particle size than day 1. In agreement, Karumendu et al. (2009) concluded that particle size could be used to detect differences in myofibrillar fragmentation of lamb meat due to aging. Particle size was also used to study the textural properties of wooden breast chicken muscle (Soglia et al., 2017), and fibrosis might have contributed to the observed larger particles in the superficial layer of wooden breast samples. Particle size measurements are thus able to reflect protein fragmentation (proteolysis) and well as protein cross-linking and aggregation.

The proteolysis of structural proteins was mainly investigated by monitoring degradation of desmin, a key protein that helps to link myofibrils to each other and myofibrils to the sarcolemma. Degradation of desmin would lead to a loosened myofibrillar structure and therefore reduced proteolysis may contribute to the tougher meat texture. In study I, however, desmin degradation was not affected by oxygen concentration in MAP (Fig. 6 & Fig. 7, study I).

Desmin is a known substrate for μ-calpain, which is believed to be one of the main proteolytic enzyme in meat. The oxidation-induced inactivation of μ-calpain is the basis for the hypothesis that oxidation reduces proteolysis (Rowe et al., 2004b). In this thesis, μ-calpain was not inactivated by oxygen in MAP (Fig. 21), which together with the observation that desmin degradation in aged samples was not affected by oxygen supports that reduced proteolysis was not responsible for the larger shear force in high oxygen packaged samples. Similarly, no effects of high oxygen MAP on degradation of desmin and autolysis of μ-calpain was previously reported by Kim et al. (2010). However, Chen et al. (2015) found that high oxygen MAP inhibited the autolysis of μ-calpain and degradation of desmin. It should be noted that Rowe et al. (2004b) used irradiation which may be much stronger than oxygen in terms of oxidative stress. In addition, the time the oxidation was introduced might be important as μ-calpain activity disappears quickly during postmortem storage.

Based on results from study I, a hypothesis is proposed for the relationship between oxygen concentrations, shear force and protein oxidation in meat packaged with oxygen (Fig. 22):

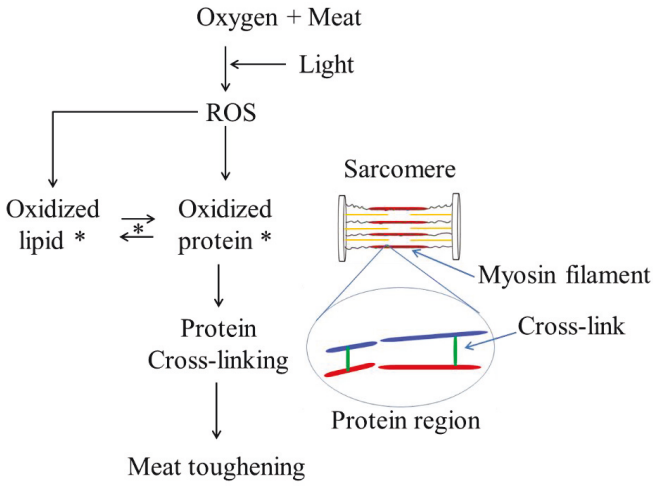

Fig. 22. Hypothesis for the relationship between oxygen concentrations in modified atmosphere packaging and toughening of meat. In the illustration of the sarcomere structure Z-disks are shown with attached actin filaments. Titin spans the sarcomere and is connected to the myosin filaments. Blue and red colors in protein region represent protein molecules which can be two myosin molecules or myosin and titin. ROS: reactive oxygen species. Free radicals are represented by * (Fig. 8, Study I).

ROS is generated in meat during storage in the presence of oxygen and light, and the generated reactive species react with other meat components leading to oxidized lipids and proteins. Free radicals may transfer between lipids and proteins while one of the consequences of oxidized proteins is to form protein cross-links, and protein cross-linking subsequently lead to meat toughening as also suggested by Lund et al. (2007). As myosin molecules locate close to each other and also close to titin, cross-links may be formed between myosin molecules or myosin and titin. Study I confirms that MHC is involved in protein cross-linking and Kim et al. (2010) suggested cross-linking between myosin and titin in high oxygen packaged beef.

A tougher meat texture was also observed in study II as the hardness of patties made from minced meat in oxygen-containing MAP (20-80% oxygen) was greater than 0% oxygen cooked at 55 ºC and 60 ºC (Table 3, study II). Unlike in meat, there is no general agreement on the effect of protein oxidation on

textural properties of myofibril gels (Table 1). The microstructure that determines the textural properties of meat and myofibrillar gels is very different (Tornberg, 2005).

6.2.2. Changes in water-holding

As summarized in Table 3 & 4 in section 2.3.2, there is no general agreement on the effect of oxidative conditions on water-holding. In this thesis, no effect of oxygen concentration on purge loss or cook loss of porcine LD muscle has been found (Table 6), but cook loss of beef patties made from high oxygen packaged minced meat was generally higher (Table 7). Tornberg (2005) pointed out that the structural origins of water-holding in whole meat and highly minced meat is different. In the whole meat, a crucial factor is shrinkage and swelling of myofibrils (Offer & Knight, 1988), while in minced meat, the gel-forming ability of meat proteins is important (Hermansson, 1986).

This thesis focus on effect of protein oxidation on water-holding of fresh meat. In study III, a model system with myofibrils oxidized in HClO showed generally increased water-holding of myofibrils following oxidation (Fig. 5, study III). The increased water-holding agrees with previous reports that mild oxidation enhance meat hydration (Delles, Xiong, & True, 2011; Liu et al., 2011). However, 'mild oxidation' is not well defined and the hydration measurements were done in the presence of high salt and pyrophosphate which may interact with oxidation on water-holding. Bertram et al. (2007) studied the water functionality of oxidized myofibrillar proteins using NMR, and they found a generally reduced T_2 relaxation time upon oxidation. A reduced relaxation time indicates increased water mobility and therefore imply reduced water-holding. However, no direct proof that oxidation decreased water-holding was obtained.

Generally, incubation of myofibrils with HClO led to increased protein oxidation (Fig. 1 & 2, study III), increased particle size (Fig. 3, study III), loss of histidine residues (Table 2, study III), shift of pI to more acidic area in IEF gel (Fig. 4, study III). Taking these results together with the increased water-holding (Fig. 5, study III), a hypothesis that oxidation-induced changes of water-holding is determined by the balance between promoting factors (such as increased filament net charges) and inhibiting factors (formation of structural constraints) is proposed (Fig. 23).

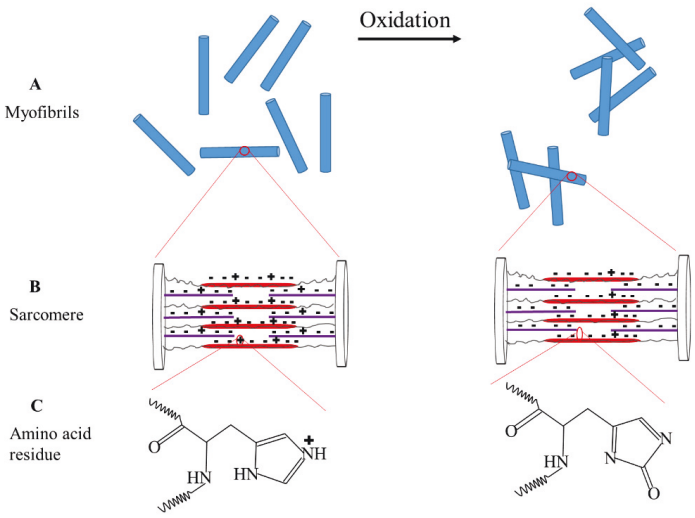

Fig. 23. Model for the effect of myofibrillar protein oxidation on filament charges, protein aggregation and water-holding. Oxidation leads to two major changes with opposite effects on water-holding: 1) Oxidation leads to aggregation of myofibrils which tends to decrease water-holding (A); 2) Conversely, within the sarcomere an increase in net negative charges of both myosin and actin filaments tends to improve water-holding (B). The change in filament charges on amino acid level is illustrated in (C), where a positively charged histidine residue forms 2-oxo-histidine following oxidation and loses its positive charge, and thereby causes an increase in the net negative charges of myofilaments (Fig. 6, Study III).

This hypothesis is based on the general belief that swelling or shrinking of myofibrils largely determines water-holding of meat and that the degree of swelling or shrinking depends on the filament net charges and transverse cross-linkages (Hamm, 1972; Offer and Knight, 1988).

Formation of cross-links limit the swelling of myofibrils, as also supported by Liu et al. (2009), where disulfide bonds were identified as one of therestricting factors for the swelling of oxidized myofibrils during brine irrigation. In study III, the improved water-holding following oxidation suggests that other factors than protein cross-links exert stronger opposite effects on water-holding.

Increased filament net charges is linked to better water-holding (section 2.3.2.1). As protein charges originates from charged amino acid sidechains, oxidative modifications of the sidechains may alter protein charges. Among the charged amino acid residues, lysine, arginine and histidine are prone to protein carbonylation (Stadtman, 1993) and thereby also loss of the positive charges that they carry, which hence affect filament net charges. Oxidation has previously been suggested to modify the charge

distribution on myofilaments and thereby influence water-holding in meat (Estévez et al., 2011; Utrera & Estévez, 2012).

The oxidation-induced changes in protein net charges was evaluated by IEF gel electrophoresis, and the pI of solubilized, oxidized myofibrillar proteins were generally observed to be lower than that of the non-oxidized control (Fig. 4, study III). This agrees with Baraibar et al. (2011) who reported that some proteins in human myoblasts shifted towards more acidic pI upon oxidative stress. In the IEF gel, bands that shifted towards a lower pI were mainly identified as myosin heavy chain, myosin light chain, actin and tropomyosin (Fig. 4, study III). Those proteins locate in either myosin filaments or actin filaments and account for approximately 73% of the myofibrillar proteins, and the results therefore suggests that the isoelectric point of myofilaments were lowered following oxidation. In study III, the pH was around 5.5 while the average pI of meat proteins is around 5.0 (Hamm, 1972). As a lower pI lead to a larger difference between pI and pH, an increase of protein net negative charges will be a consequence.

A limitation of IEF gel electrophoresis is that this technique only allows analyses of solubilized proteins. To avoid just to study a fraction of the oxidized myofibrils, an amino acid analysis was performed on the acid hydrolysates of the myofibril pellets (Table 2, study III). Histidine was found to decrease with oxidation and it was hypothesized that histidine side chains were partly carbonylated into 2-oxo-histidine and thereby lost their positive charges upon oxidation. The observed loss of histidine supports the hypothesis that oxidation leads to an increase of net negative charge on actin and myosin filaments. One shortcoming of the analytical approach used to determine the amino acid composition following oxidation is that HCl hydrolysis destroys cysteine, methionine and tryptophan, which are highly oxidizable amino acids. These amino acids are therefore also highly relevant to evaluate, and it would be of interest to further study the changes in amino acid composition using alternative hydrolysis methods. In addition to this, analysis for oxidation products is often more sensitive for detection of oxidative damage, as it is often difficult to detect small losses of amino acids. Analysis of specific oxidation products such as 2-oxo-histidine, AAS and GGS could thus potentially add valuable information.

In addition to the increase of net negative charges, the positively charged histidine residues are strong chaotropes and loss of chaotropes through histidine carbonylation are also hypothesized to be linked to reduced water-holding.

6.2.3. Effect of protein oxidation on color of raw and cooked meat color

High oxygen MAP is mainly used to maintain the attractive bright red color of fresh red meat. However, high oxygen levels promote lipid oxidation, which can have adverse effect on color (Faustman, Sun, Mancini, & Suman, 2010). The effect that oxygen helps to maintain the red color was observed in minced beef stored under 40-80% oxygen (Table 2, study II), but not in porcine LD muscle after chilled storage for 14 days in MAP with oxygen (Table 5). Several studies have shown that high oxygen MAP only created the desirable red color of meat during early display, but the rate of discoloration was more rapid in high oxygen MAP (Grobbel, Dikeman, Hunt, & Milliken, 2008; John et al., 2005; Kim et al., 2010; O'Grady, Monahan, Burke, & Allen, 2000).

According to Sørheim & Høy (2013), the meat pigment would be predominantly in the form of oxymyoglobin immediately after mincing. If the meat is cooked when the major part of myoglobin is in the oxygenated form, premature browning occurs. In study II, the patties of day 0 did not show premature browning (Fig. 1, study II), possibly due to fact that after mincing the meat was kept for approximately 2 h before cooking, and therefore myoglobin in the inner part of the minced meat patties likely turned into more heat-stable deoxymyoglobin. The presence of undenatured deoxymyoglobin at lower cooking temperatures was suggested by the phenomenon that some of the cooked patties gradually developed the red color on the cut surface, likely due to blooming upon exposure to atmospheric oxygen.

After storage in MAP for 6 days, the redness of cooked patties generally decreased with increasing oxygen concentrations (Table 3, study II), suggesting a gradual loss of deoxymyoglobin and/or an increase of metmyoglobin due to increasing oxidative stress. As oxygen concentration increases in MAP, the layer of oxymyoglobin penetrates deeper to the meat and thus decreases the content of deoxymyoglobin (Mancini & Hunt, 2005). Increased oxidative stress also promotes lipid and protein oxidation, where lipid oxidation can facilitate the formation of metmyoglobin (Faustman, Liebler, McClure, & Sun, 1999; O'Grady, Monahan, & Brunton, 2001) and protein oxidation may promote protein denaturation, and the denatured proteins may co-precipitate with myoglobin (Ledward, 1971) therefore reduce the heat-stability. Oxidative stress may also reduce the activity of metmyoglobin reductase, which leads to the formation of metmyoglobin. With increasing cooking temperatures, the internal color of patties turned to more brown as expected. And eventually, the patties became brown in all packaging groups as the temperature became high enough to denature deoxymyoglobin.

Previous studies have shown that high oxygen packaging was able to cause premature browning. In study II, it was interesting to observe premature browning already at a relative low oxygen concentration of 20%. This information could be of importance as this concentration is similar to atmospheric oxygen composition.

7. Conclusions

The current thesis provided a better understanding of effects of oxidative conditions on protein oxidation in meat and its relationship with meat quality traits: texture, water-holding and color.

Increasing concentrations of oxygen (0, 20, 40, 60, 80%) in MAP led to increased protein oxidation and lipid oxidation, and the increased concentrations of HClO (0, 1, 5, 10 mM) also led to greater protein oxidation in isolated myofibrils.

Shear force of pork slices and formation of protein cross-links were generally larger in oxygen-containing MAP (20–80% oxygen) compared to 0% oxygen, while desmin degradation was not affected by packaging. Therefore, the results revealed a meat toughening effect and suggested that the mechanism is through oxidation-mediated protein cross-linking rather than through reduced proteolysis. A meat toughening effect was also observed in minced beef evidenced by greater hardness of cooked patties in oxygen-containing MAP.

In addition to meat toughening, oxygen in MAP led to premature browning when minced beef was cooked at lower temperatures (55-65 ºC), and this was observed already at a relative low oxygen concentration of 20%. As meat toughening negatively affect meat tenderness and premature browning may lead to insufficient cooking of meat (a safety risk that pathogens in meat may survive), oxygen in MAP for fresh meat is not recommended.

In this thesis, a new hypothesis is proposed concerning the effect of myofibrillar protein oxidation on filament net charges, protein aggregation and water-holding:

Upon oxidation, an increase in particle size of myofibrils is linked to formation of protein cross-links and aggregates which act to inhibit swelling of myofibrils. An opposite effect from electrostatic charges is simultaneously promoting swelling. The overall net negative charges in myofibrillar proteins including myosin, actin and tropomyosin increase with oxidation which leads to increased net negative charges on both myosin and actin filaments. More net negative charges on the myofilaments cause greater swelling pressure, which is linked to better water-holding. The overall result of protein oxidation on water-holding is therefore a balance between promoting factors (such as increased net charges) and inhibiting factors (such as protein cross-linking and aggregation). The overall net negative charges of myofilaments provide a novel perspective to understand oxidation-induced meat quality changes.

8. Future perspectives

This thesis tested the effects of protein oxidation on meat texture, color and water-holding. One of the main findings of this thesis is that MAP with a relative low oxygen concentration (as low as 20%) promoted protein oxidation and led to meat toughening and premature browning in cooked meat. This information is useful for producers and users since this oxygen concentration (20%) is similar to atmospheric air. Another major finding is that oxidation shifted the isoelectric point of major myofibrillar proteins to more acidic values and the histidine content decreased. This lead to a new hypothesis that protein oxidation leads to a decrease of positive charges through carbonylation of histidine residues. The oxidation-induced change in net charge was suggested to be linked to water-holding of meat in the thesis, and this view could provide a novel perspective to understand other oxidation-induced meat quality changes as well. Histidine contributes to protein charges and meat buffering capacity, it is also prone to carbonylation. Therefore, histidine oxidation requires more focus in the field of meat science.

Finally, the work presented in the thesis has given rise to new questions that can be addressed in future studies:

1) While myofibrils is a good and relative simple model for study meat water-holding, sarcoplasmic proteins and the extracellular space are also important determining factors for water-holding. Further studies with focus on water-holding can include minced meat, small meat pieces or meat products.

2) It is presently unknown how relevant it is to study HClO-mediated protein oxidation in meat and meat systems, although HClO is a known strong oxidant and it can be produced *in vivo*. It would be of interest to extend this study to other oxidation systems, such as the Fenton reaction, to investigate if similar results can be obtained.

3) The relationship between lipid and protein oxidation is very complicated and the information in the literature is very limited. This topic thus requires further exploration.

4) Protein oxidation often leads to decreased solubility. Most analytical methods require the solubilization of proteins. There is a risk that only the fraction of solubilized proteins is studied. Methods which can bring more proteins into solution, if not all, would be very useful in the research field of protein oxidation.

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